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Non-apoptotic Fas functions are critical in the control of T cell expansion

TESIS DOCTORAL

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ABBREVIATIONS

AICD	Activation Induced Cell Death
Akt	protein kinase B
ALPS	Autoimmune lymphoproliferative syndrome
APC	antigen presenting cell
BCL-10	B-cell lymphoma/leukemia 10
BFA	brefeldin A
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
bVAD	biotin-valyl-alanyl-aspartyl-fluoromethylketone
CARMA-1	Caspase recruitment domain-containing protein 11
CBM	complex composed of CARMA-1, BCL-10 and MALT-1
c-FLIP	cellular FLIP
CFSE	Carboxyfluorescein diacetate succinimidyl ester
ConA	Concanavalina A
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DMSO	Dimethyl sulfoxide
DN	double negative
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FADD	Fas-Associated protein with Death Domain
FACS	Fluorescence-activated cell sortin
FasL	Fas ligand
FCS	fetal calf sera
FITC	fluorescein isothiocyanate
FLIP	FLICE-like Inhibitor Protein
gld	generalized lymphoproliferative disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IETD	Z-I-E(OMe)-T-D(OMe)-fluoromethylketone
IFN	Interferon
Ig	Immunoglobulin

IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK	IκB kinase
JNK	c-Jun N-terminal kinase
LAT	linker for activation of T cells
Lck	Lymphocyte-specific protein tyrosine kinase
lpr	lymphoproliferation
MALT-1	Mucosa-associated lymphoid tissue lymphoma translocation
MAPK	Mitogen-Activated Protein Kinase
MHC	Major histocompatibility complex protein 1
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI3K	Phosphatidylinositol 3-kinases
PKC	Protein kinase C
PMA	phorbol myristate acetate
rIL-2	recombinant interleukin-2
rpm	revolution per minute
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
SMAC	Supramolecular activation complex
Src	sarcoma tyrosine kinase
TCR	T cell receptor
Tg	transgenic
Th	helper T cell
T[H3]	tritiated thymidine
TNF	tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
ZAP-70	ζ chain-associated protein kinase 70 kDa
zVAD	benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone

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Supplement

The immune system is regulated by a complex set of biological processes, mediated by a variety of specialized molecules; it protects the organism against pathogens and against its own damaged, potentially dangerous cells. The first line of defense consists of the components of the innate immune system, which provide an immediate but non-specific response. This defense is provided principally by phagocytes (macrophages, dendritic cells and neutrophils) (Medzhitov et al., 2007). In addition to identifying and eliminating pathogens, innate immune cells are important mediators in the activation of the adaptive immune response. This second type of response is triggered by pathogens that successfully escape the control of the innate system, and is characterized by high specificity for antigens. The adaptive immune response can be classified on the basis of the immune components that participate in it, which consist of the cellular immune response (mediated by CD4+ and CD8+ T cells) and the humoral immune response (mediated by B cells).

The T cells of the adaptive immune system play a crucial role in defending the organism against pathogens. Initial stimulation by foreign antigens presented by antigen-presenting cells (APC) induces activation and clonal proliferation of T cells, to generate effector cells that clear pathogen from the body. This process, termed clonal expansion, is needed to produce a sufficient number of pathogen-specific effector T cells. The effector functions of CD4+ T cells consist of cytokine and chemokine production, which augment the immune response at the focus of infection. Based on the pattern of cytokines produced, CD4+ T cells can be classified as Th1 (type 1 T helper cells), which secrete IL-2 and IFNγ, Th2 (type 2 T helper cells), which secrete IL-4 and IL-5 and Th17 (T helper 17 cells), which produce IL-17 and IL-22. In addition to these populations, whose effector functions are directed toward pathogen elimination, another CD4+ T cell subtype has been identified, the CD4+ regulatory T cells (Treg). These cells (phenotypically defined as CD4+CD25+CD127^{low}Foxp3+) produce suppressor cytokines such as IL-10 and TGF-β, and suppress T cell functions to avoid chronic activation and autoreactivity of T cells in the periphery (Aluvihare et al., 2006; Bluestone et al., 2003).

At difference from CD4+ T cells, whose effector mechanisms are based mainly on cytokine secretion, CD8+ T

cells act as cytotoxic T cells that directly kill target cells infected with virus or intracellular microorganisms.

After antigen clearance, the clonally expanded activated T cell population must be eliminated to maintain T cell homeostasis and to prevent development of autoimmunity and hyperproliferative diseases; this process is called the contraction phase (Krammer et al., 2007; Strasser et al., 2004). Although Fas-mediated apoptosis was considered the main mechanism involved in this process, *in vivo* regulation of the contraction phase and the contribution of the distinct death pathways (Fas- and Bim-driven) is still poorly understood (McKinstry et al., 2010). Due to the absence of pathogen-derived antigens, T cells stop receiving activation and survival signals from the APC. Only a few T cells previously exposed to antigen are able to survive and develop into memory T cells, which provide a more rapid response after secondary challenge with the same antigen (Murali-Krishna et al., 1998; Blattman et al., 2002; Sprent et al., 2002; Surh et al., 2006).

T cell homeostasis is mediated by the balance between cell death and proliferation. This balance is characterized by rapid clonal expansion of antigen-reactive lymphocytes, followed by apoptosis of activated cells. The misregulation of apoptosis or proliferation of apoptosis-surviving T cells can lead to a number of immune diseases (Arias et al., 2007).

Tolerance and autoimmunity

One characteristic of the immune system is its capacity to recognize and respond only to external antigens. The absence of a response to the organism's own antigens is called self tolerance; loss of the ability to distinguish between self and non-self leads to immune responses against autoantigens and to development of autoimmune diseases.

Self tolerance is maintained through central and peripheral mechanisms. Central tolerance, which takes place during the maturation process in the thymus, ensures elimination of lymphocytes able to recognize high affinity autoantigens presented in the thymus. This selection process is termed negative selection. There is also positive selection, which ensures that the TCR (T cell receptor) is able to recognize its cognate ligand, the peptide-binding major histocompatibility complex (MHC). T cells whose TCR does not fulfill this requirement and thus do not receive survival signals are eliminated through apoptosis in a process called "death by neglect". Positive and negative selection together allow the thymus to produce self-tolerant, functionally competent T cells, in order to establish immunity against foreign pathogens (Sprent et al., 2002; Gascoigne et al., 2011; Metzger et al., 2011). Recent studies nonetheless suggest that not all thy-

mocytes with high affinity for self antigen are deleted by negative selection in the thymus; some survive and undergo non-deletional central tolerance that leads to generation of immunosuppressor CD4+ T cells known as naturally-occurring regulatory T cells (Treg; Sakaguchi et al., 2003; Liu et al., 2006).

The majority of autoreactive T cells is eliminated during the central tolerance process. However, not all endogenous autoantigens are present in the thymus and a small percentage of T cells with autoreactive capacity is thus released to the periphery (Burns et al., 1983). Here, the second level of control permits limitation of the autoreactive T cell response during encounter with autoantigens in tissues outside the thymus; this is peripheral tolerance. This process is based on a variety of complex mechanisms, such as immunological ignorance, deletion, inhibition or suppression of autoreactive clones (Walker et al., 2002; Abbas et al., 2003; 2004).

Immunological ignorance is caused by the physical separation between T cells and antigens, such as that seen in the blood-brain barrier, or by antigen levels insufficient to activate T lymphocytes. Another mechanism is clonal anergy, which consists of antigen presentation in the absence of necessary costimulation. The failure of full T cell activation leads to functional inability to respond, or to cell death by apoptosis.

The mechanisms of immunosuppression include several immunoregulatory cell populations whose basic characteristic is the ability to secrete immunosuppressor cytokines, such as IL-4, IL-10 and TGF-β. Naturally-occurring Treg (CD4+CD25+CD127^{low}Foxp3+) are among these cells, but there are also other immunoregulatory subsets, including NK-T cells, CD8+CD28- T cells, T$\delta\gamma$ cells, and DN (double negative; CD4-CD8-) T cells.

Another important mechanism that aborts T cell activation after autoantigen recognition is the programmed death of T cells by apoptosis. It has become evident that elimination of activated T cells is the main mechanism for ensuring tolerance in the periphery (Krueger et al., 2003). Apoptosis mediated by Fas, a member of the tumor necrosis factor (TNF) receptor family, is fundamental for T cell homeostasis and tolerance induction. This apoptosis system was considered the major death pathway in the termination of immune response, and is well established *in vitro* in human and murine systems (Dhein et al., 1995; Suda et al., 1997). Nevertheless, some *in vivo* studies questioned the prominence of its function in this process (Strasser and Pellegrini, 2004; Lohman et al., 1996; Pellegrini et al., 2003). In addition to Fas/FasL signaling, various reports indicated that Bim has a central role in T cell death induction (Pellegrini et al., 2003; Hughes et al., 2008).

Activation-induced cell death (AICD) is the *in vitro* process of cell death induction of already-activated T cells by restimulation of the TCR. In this experimental

model, naïve T cells are first activated with antigens or mitogens, expanded in culture medium containing IL-2, and re-stimulated in a TCR-dependent manner (Scheme 5, Material and Methods). As a consequence, the majority of reactivated T cells die through apoptosis. Extensive studies demonstrated that Fas can drive the AICD observed *in vitro.* Following restimulation, upregulation of Fas ligand (FasL) takes place, and the Fas/FasL interaction triggers activation of initial and effector caspases that lead to cell death (Krammer et al., 2000; Green et al., 2003; Strasser et al., 2000; Nagata et al., 1995). Sensitivity to AICD is strictly controlled by the activation status of T cells (Klas et al., 1993; Peter et al., 1997). Its initiation requires IL-2 exposure prior to secondary TCR stimulation (Walker and Abbas, 2002). Apart from leading to AICD sensitization, IL-2 is also an essential factor in T cell proliferation (Lenardo et al., 1991).

The Fas/FasL apoptotic system

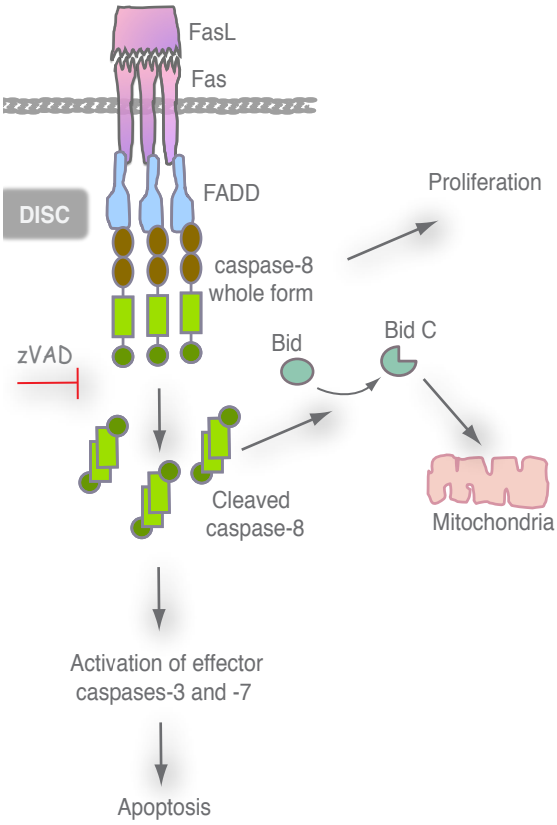
Fas was initially characterized as a cell membrane receptor with the capacity to induce cell death of cells treated *in vitro* with monoclonal anti-Fas antibodies (Ogasawara et al., 1993). Fas, also known as CD95, TNFR6, DR1 or APO-1, is expressed on the plasma membrane as a pre-associated homotrimer. As mentioned above, Fas is a member of the TNF family, as it has the three extracellular cysteine-rich domains characteristic of this superfamily (Krammer et al., 2000; Green et al., 2003; Krueger et al., 2003). A common feature of death receptors, including TNF family members, is the presence in their cytoplasmic tail of the death domain (DD), a conserved 80-amino-acid region that serves as a platform for death-inducing signaling complex (DISC) formation (Wajant et al., 2003; Delalande et al., 2008).

FasL (CD95L, APO-1L, CD178) is a TNF family ligand and is also assembled at the plasma membrane in homotrimer form (Li-Weber et al., 2003). Proteolysis can generate soluble forms of this ligand composed of extracellular protein fragments, but a recent report indicated that only the membrane-bound FasL form is essential for apoptosis induction (O'Reilly et al., 2009).

Following binding of FasL, Fas undergoes conformational changes that allow recruitment of the adaptor molecule FADD (Fas-associated death domain protein) through homotypic interaction of death domains present in both molecules. This in turn leads to the recruitment of initiator caspase-8 and formation of the DISC (Kischkel et al., 1995; Peter et al., 2003). The interaction of FADD and caspase-8 is mediated through binding of death effector domains (DED) found in both proteins (Tibbetts et al., 2003; Bernhart et al., 2003). A number of trimeric Fas receptor

units, each in association with DISC, form aggregates called SPOTS (signaling protein oligomeric transduction structures; Siegel et al., 2004). The oligomerized receptors must be internalized through clathrin-dependent endocytosis; this allows further recruitment of DISC components and permits the local caspase-8 concentration necessary for its autoprocessing. The receptor internalization is an obligatory step, since its inhibition impairs full DISC formation and apoptosis (Lee et al., 2006). As a result of auto-cleavage, the capase-8 large (p18) and small subunits (p10) are released, and reassemble to form heterotetramers that consist of two large and two small subunits; this is the enzymatically active caspase-8 form. This activated caspase-8 is released into the cytoplasm, where it targets downstream effector caspases such as caspase-3 and -7, and triggers apoptosis (Boatright et al., 2003; Chang et al., 2003). Interestingly, the whole (unprocessed) caspase-8 form is associated with lymphocyte proliferation (Lamkanfi et al., 2007; Bidere et al., 2006a, Scheme 1).

FLIP (FLICE-inhibitory protein) is one of the potent negative regulators of the Fas signaling pathway, at the level of DISC; it is a caspase-8-like protein that has two death effector domains and a pseudo-caspase-8-like domain, but lacks enzymatic activity (Budd et al., 2006). In low concentrations, cFLIP (cellular FLIP) acts as a positive regulator of caspase-8 activation. Increased amounts of cFLIP nonetheless inhibit caspase-8 activation, probably by replacing caspase-8 in the DISC and thus dampening apoptosis (Chau et al., 2011; Chang et al., 2002; Irmler et al., 1997; Katoka et al., 1998).



Scheme 1. Apoptotic pathway
Binding of Fas by FasL induces Fas conformational changes that lead to FADD recruitment, which in turn recruits caspase-8 and results in formation of DISC (death-inducing signaling complex). Within DISC, caspase-8 is autoprocessed, and active cleaved caspase-8 is released to the cytoplasm, where it can target downstream effector caspases and induce apoptosis. Caspase-8 can also amplify the apoptotic signal by cleaving Bid. The cell death inhibitor zVAD blocks apoptosis at the level of DISC, impeding caspase-8 autoprocessing.

Autoimmune disease development in B6/*lpr* mice

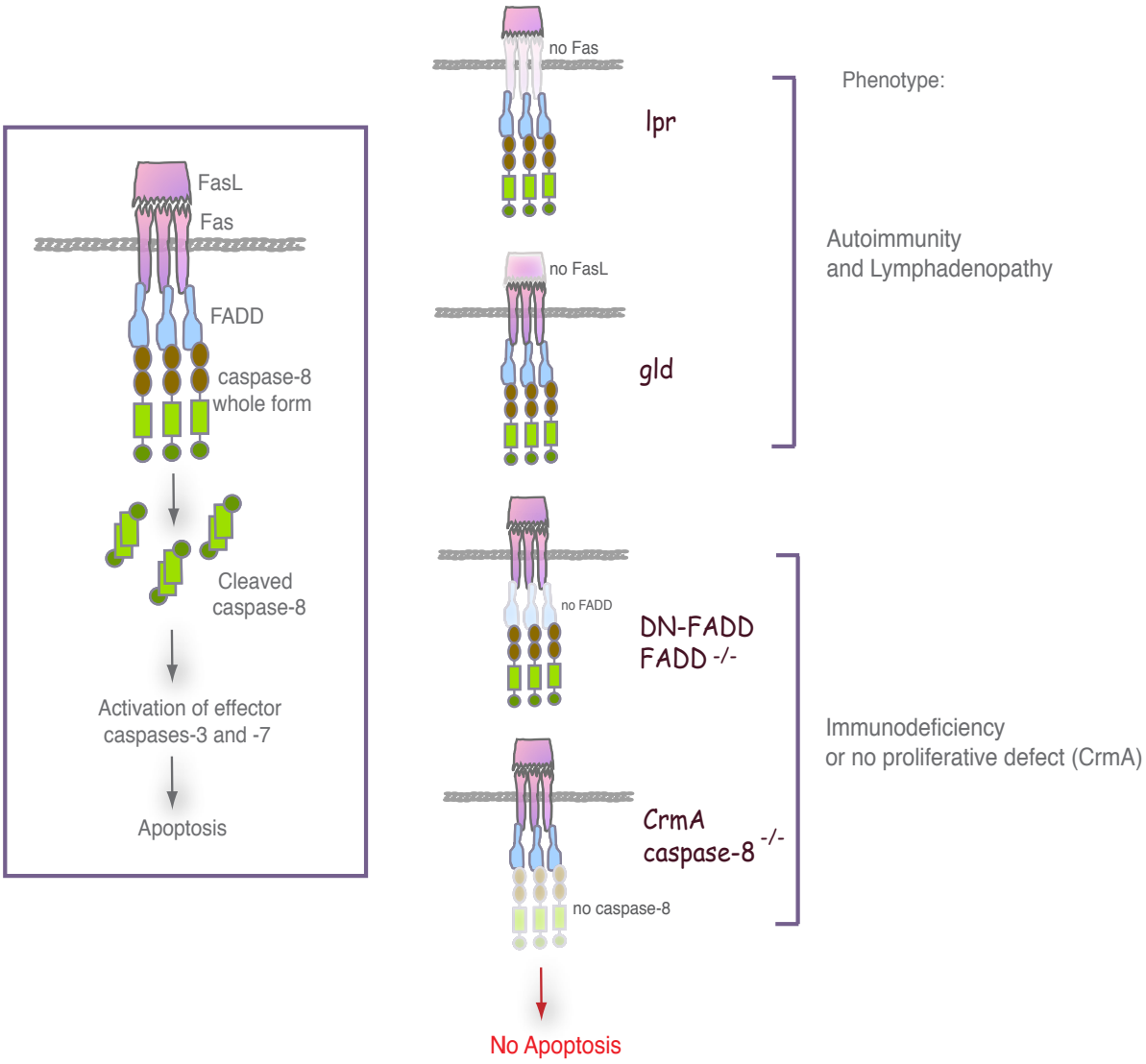
B6/*lpr* (from lymphoproliferation) mice show Fas deficiency due to spontaneous retrotransposon insertion into intron 2 of the *Fas* gene. This leads to defective apoptosis signaling and development of autoimmune disease, characterized by lymphadenopathy and splenomegaly. These abnormalities originate from the accumulation of DN T cells (TCRab+CD4-CD8-B220+), which are rare components of the peripheral T cell repertoire. Accumulation of CD4+ memory T cells (CD44high/CD62Llow) and IgG+ B cells is also observed. In addition, Fas-deficient mice have elevated levels of anti-DNA antibodies in serum, immune complex formation in tissues, and mild glomerulonephritis. This autoimmune phenotype is much more severe in mice on the MRL background (MRL/*lpr*), in which kidney failure and death are the final consequence of the disease (Walker and Abbas, 2002).

Lack of Fas-triggered apoptosis in activated T cells of B6/*lpr* mice was suggested to be a direct cause of lymphadenopathy development (Watanabe-Fukunaga et al., 1992). However, the etiology of autoimmune disease and lymphadenopathy development in B6/*lpr* mice remains elusive, since the *in vitro* T cell apoptotic defect is not always associated with reduced apoptosis *in vivo* (Walker and Abbas, 2002). Moreover, cell death defects themselves do not explain the B6/*lpr* phenotype. In the absence of other apoptotic pathway components such as FADD (as in FADD$^{-/-}$ mice and transgenic mice that overexpress a dominant negative form of FADD) and caspase-8 (in humans with an inherited genetic deficiency of caspase-8 and in mice with caspase-8 deficiency in T cells), lack of apoptosis is accompanied by immunodeficiency (Zhang et al., 1998; Newton et al., 1998; Newton et al., 2001; Chun et al., 2002; Salmena et al., 2003). On the other hand, transgenic mice that overexpress the caspase-8 inhibitor CrmA in T cells, and thus lack apoptotic signaling, show no phenotypic abnormalities and do not develop lymphadenopathy (Smith et al., 1996, Scheme 2).

Mouse models with abnormal FADD and caspase-8 expression or function showed that the origin of autoimmune disease associated with impaired Fas signaling is more complex than simply the result of defective cell death. Lack of FADD and caspase-8 in various mouse models and human pathologies does not reproduce the phenotype of Fas deficiency, although there is a clear resistance to apoptosis *in vitro*. These observations question the apoptosis defect due to Fas deficiency as the direct cause of autoimmunity development, and reveal a gap between the known *in vitro* properties of Fas and the *in vivo* phenotypic abnormalities observed in

its absence, including lymphadenopathy development and lupus-like disease.

In addition to autoimmune disease development in B6/*lpr* mice, T cells from their lymph nodes hyperproliferate *in vivo* (Balomenos et al., 1997; Fortner and Budd, 2005); this characteristic of Fas-deficient mice also remains unexplained. A similar hyperproliferative T cell phenotype is observed in patients with ALPS (autoimmune lymphoproliferative syndrome; Straus et al., 1999), an autoimmune disease also characterized by defects in Fas-mediated apoptosis, accompanied by lymphadenopathy, splenomegaly, DN T cell accumulation, elevated serum IgG and autoantibodies, and high risk of lymphoma development. Glomerulonephritis, autoimmune hemolytic anemia and autoimmune neutropenia are also found in some patients. In most cases, development of this disease takes place early in life, usually before five years of age (Biedere et al., 2006b; Neven et al.,



Scheme 2. Summary of the immunological consequences associated with absence of Fas, FasL, FADD or caspase-8

2011). Understanding the nature of the T cell hyperproliferation observed in B6/*lpr* mice would thus be of great value in the context of ALPS and of other autoimmune diseases characterized by uncontrolled lymphocyte expansion.

The importance of T cell hyperproliferation in the development of lymhadenopathy and lupus-like disease was previously shown by our lab (Arias et al., 2007). Mice deficient in the cell cycle inhibitor p21 develop autoimmunity, indicating the relevance of lymphoproliferation in autoimmune disease development. In this thesis, we show that inhibition of *in vivo* T cell hyperproliferation by p21 overexpression greatly reduces lymphadenopathy in B6/*lpr* mice.

NF-κB activation after TCR stimulation depends on whole caspase-8 molecule

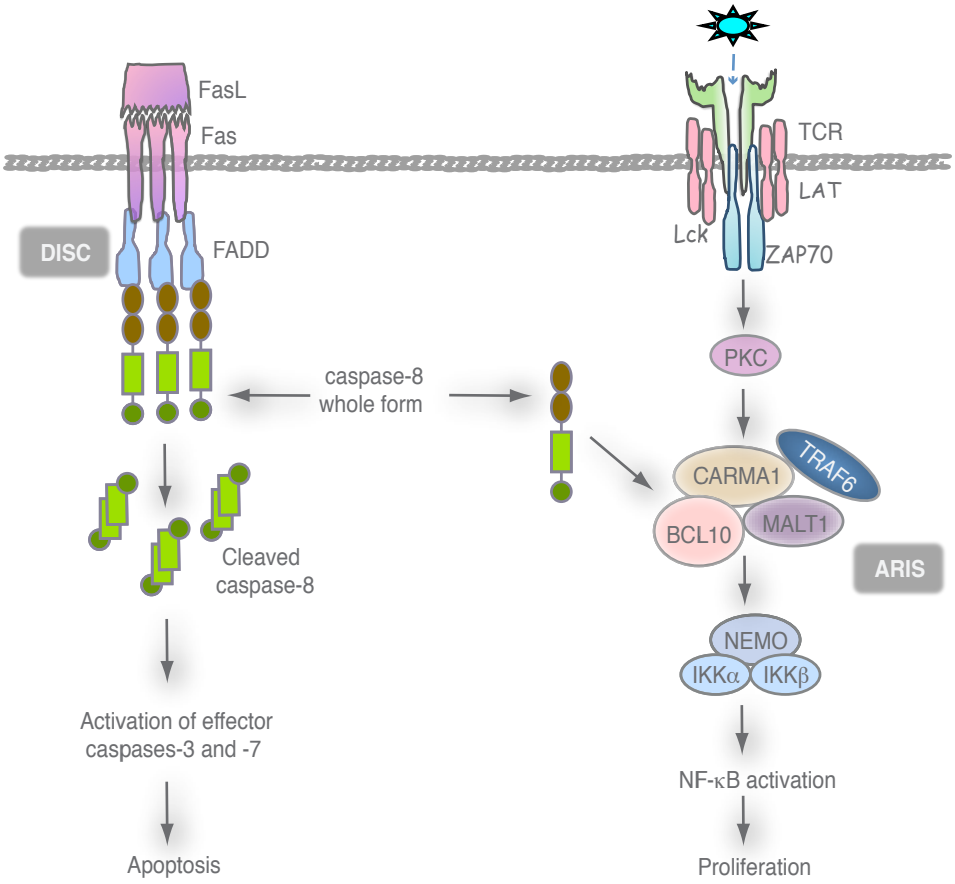
The intracellular aspartic-specific cysteine protease caspase-8 initiates death signaling for the extrinsic apoptotic pathway, which is triggered by several death receptors of the TNF receptor family such as TNFR1, CD95, TRAIL, DR3 and DR6 (Ashkenazi et al., 1998). Caspase-8 can also amplify the apoptotic signal by activating the intrinsic apoptotic pathway through the cleavage of BID (BH3-interacting domain death agonist) (Luo et al., 1998, Scheme 1).

It is now well established that, in addition to promoting apoptosis, active casapse-8 is essential for T cell activation and proliferation after TCR stimulation, and that its activity is necessary for NF-κB activation (Chun et al., 2002; Salmena et al., 2003; Siegel 2006; Lamkanfi et al., 2007). In its cleaved form, caspase-8 acts as a pro-apoptotic molecule, whereas its role in lymphocyte proliferation is associated with the activity of the whole form of the molecule (Lamkanfi et al., 2007; Bidere et al., 2006a, Scheme 4). Inhibition of caspase-8 activity *in vivo* and *in vitro* in human and murine CD4+ T cells leads to reduced proliferation and IL-2 production after primary TCR stimulation (Kennedy et al., 1999; Falk et al., 2004; Mistra et al., 2005), making caspase-8 indispensable for a normal lymphocyte proliferation.

After T cell-APC contact, receptors and intracellular proteins implicated in a number of molecular pathways assemble into the immunological synapse (IS), where the supramolecular activation cluster (SMAC) is formed. The TCR is located within the central zone of the cluster (cSMAC), where other signaling molecules including PKCθ and CARMA-1 are also found (Saito et al., 2010; Sun et al., 2000; Altman and Villalba 2003). In this context, PKCθ phosphorylates CARMA1, which induces BCL10 and MALT1 recruitment and formation of the CBM (CARMA1, BCL10, MALT1) complex (Matsumoto et al., 2005;

Sommer et al., 2005). Recruitment of caspase-8 leads to formation of the receptor-induced signalosome (ARIS), where caspase-8 interacts physically with BCL10 and MALT1 and acquires the catalytically active conformation that promotes recruitment of TRAF6 and the IKK complex (Su et al., 2005; Bidere et al., 2006a,b; Lemmers et al., 2006; Misra et al., 2007, Scheme 3). In the absence of caspase-8, IKK recruitment to the CBM complex is diminished (Su et al., 2005). Although MALT1 was reported to activate caspase-8 directly through heterodimerization (Kawadler et al., 2008), how caspase-8 is activated to mediate NF-κB signaling remains poorly understood (Du, 2011).

The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (NEMO) (Li and Verma 2002). Activation of this complex is a key step in NF-κB signaling and T cell proliferation. In unstimulated cells, IκB inhibitors bind to NF-κB p50/p65 dimers and thus sequester them in the cytosol. These inhibitors were originally thought to retain p50/p65 dimers in the cytoplasm



Scheme 3. Involvement of the uncleaved caspase-8 in TCR-triggered NF-κB activation
Following TCR stimulation, PKCθ is activated and phosphorylates CARMA1, which induces BCL10 and MALT1 recruitment as well as formation of the CBM (CARMA1, BCL10, MALT1) complex. Recruitment of whole form of caspase-8 leads to formation of the receptor-induced signalosome (ARIS), where caspase-8 interacts physically with BCL10 and MALT1, and acquires the catalytically active conformation that promotes TRAF6 and IKK complex recruitment. Activation of the latter leads to NF-κB translocation to the nucleus, where multiple pro-proliferative genes are activated, resulting in T cell proliferation.

by masking their nuclear localization sequences (NLS), although some reports indicate that IκB inhibitors shuttle between the nucleus and cytoplasm, transporting p50/p65 from target DNA sites back to the cytoplasm (Birbach et al., 2002). IκB protein expression is directly regulated by NF-κB; this feedback regulation is believed to contribute to the rapid shutdown of NF-κB signaling. In the classical pathway, an upstream signaling event induces activation of IKKβ, which then phosphorylates IκB, releasing it from the p50/p65 dimer and targeting it for polyubiquitination and proteosomal degradation. The p50/p65 NF-κB dimer is then released and translocates to the nucleus to bind to DNA and regulate gene transcription (Li and Verma 2002; Su et al., 2005).

Upstream TCR signaling events and receptor internalization

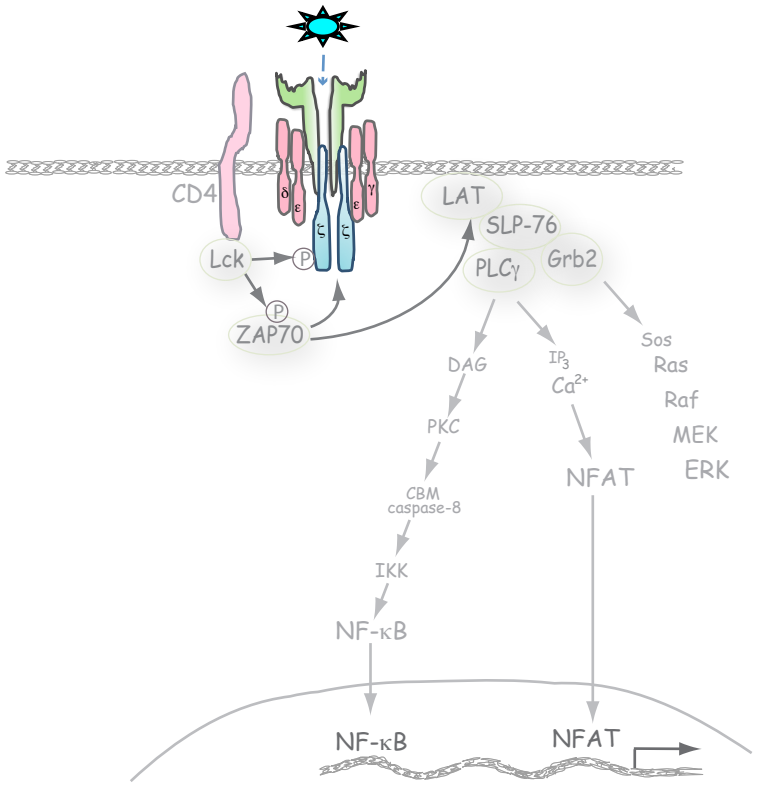
Triggering of the TCR at the T cell surface by APC or monoclonal antibodies initiates a signaling cascade of well-regulated intracellular events that leads to cell activation, proliferation, and immune functions (Cheng et al., 2011; Bécart et al., 2009; Ullman et al., 1990; Weiss et al., 1994), as well as receptor internalization and downmodulation of the TCR (Krangel 1987; Minami et al., 1987; Telerman et al., 1987, Boyer at al., 1989; Boyer et al., 1991). TCR internalization contributes to receptor desensitization, leading to transient or long-term anergy to antigen. Cai et al. suggested that the most likely function of TCR downmodulation is to protect cells from overstimulation (Cai et al., 1997); Schonrich et al. also showed that TCR downmodulation is an effective means of tolerance induction (Schonrich et al., 1991).

The TCR/CD3 complex is composed of the TCR-αβ heterodimer, which is responsible for recognition of the antigen as a peptide presented by MHC (Bjorkman et al., 1987), and of the CD3 transduction complex (Clevers et al., 1988). The latter is composed of CD3-γ, -δ, -ε subunits, which are noncovalently associated to the TCR as CD3-δε and CD3-γε doublet forms (Koning et al., 1990; Hera et al., 1991). The second transduction molecule required for surface expression of the TCR/CD3 complex is the ζ component (Klausner et ai., 1990; Irving and Weiss 1991; Wegener et al., 1992), a member of a related family of molecules that includes CD3-ζ, CD3-η and FcεRIγ (Weissman et al., 1988; Blank et al., 1989). TCR-ζ and other CD3 signaling subunits have a characteristic sequence motif for tyrosine phosphorylation, known as ITAM (immunoreceptor tyrosine-based activation motif). The cytoplasmic segment of CD3-γ, CD3-δ and CD3-ε has a single ITAM, whereas that of the TCR-ζ subunit bears three ITAM copies. As a result, each αβTCR heterodimer is functionally coupled to ten ITAM (Malissen,

2008). In addition, three protein tyrosine kinases (PTK) are associated to the TCR/CD3 complex, 1) the Src family member $p56^{lck}$, which interacts selectively with CD4 and CD8 coreceptors (Veillette et al., 1988), 2) the Src family member $p59^{fyn}$, which is associated at low stoichiometry with the TCR/CD3 complex (Samelson et al., 1990), and 3) the syk-related ZAP70, which is uniquely associated with the tyrosine-phosphorylated TCR-ζ (Chan et al., 1991).

The TCR signaling cascade is initiated by TCR binding of the MHC-bound peptide. This allows members of the Src family (Lck and, to a lesser degree, Fyn) to initiate T cell activation by phosphorylating tyrosine residues located within CD3 ITAM, and permits ZAP70 recruitment in close proximity to the activated TCR. By phosphorylating the tyrosine residue in the ZAP70 activation loop, Lck increases the kinase activity of ITAM-bound ZAP70, which in turn recruits and phosphorylates adaptor molecules such as LAT, SLAP, SLP-76, and possibly Vav1, Cbl and PLC-γ1. This leads to activation of multiple pathways, including ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), NF-κB and NFAT (nuclear factor of activated T cells), which ultimately induce cell effector functions (Davis 2002; Coombs et al., 2002; Okkenhaug and Vanhaesebroeck 2004, Scheme 4).

One of the crucial processes in turning off TCR signaling is TCR internalization and its subsequent degradation. The TCR/CD3 complex is internalized through clathrin-coated vesicles (Telerman et al., 1987; Boyer et al., 1991), a pathway used by most surface receptors, including nu-



Scheme 4. Simplified scheme of TCR signaling TCR binding results in Lck phosphorylation of tyrosine residues of the TCR. This leads to recruitment and phosphorylation of ZAP70, which in turn recruits and phosphorylates adaptor molecules such as LAT, SLP-76, PLC-γ1 and Grb2. Multiple downstream signaling pathways, including NF-κB, NFAT and ERK are subsequently activated and ultimately induce cell effector functions.

trient receptors such as transferrin (Iacopetta et al., 1988; Damke at al., 1994). In this endocytosis pathway, ligand binding leads to receptor recruitment to clathrin (present in clathrin-coated pits) through adaptors such as AP2 (CD2-associated protein) or β-arrestin1. Clathrin then polymerizes, driving infolding of the pit, which is eventually released into the cytoplasm (Doherty and McMahon, 2009). After internalization, receptors are routed to early endosomes, from which they can either be recycled to the plasma membrane or directed to the late endosomes, multivesicular bodies, lysosomes and afterwards degraded (Stenmark 2009). In the course of the last process, receptors are ubiquitinated by the Cbl family of ubiquitin ligases (c-Cbl and Cbl-b) prior to degradation (Naramura et al., 2002; Schmidt and Dikic 2005).

In resting T cells, the TCR/CD3 complex is continuously internalized and recycled to the cell surface (Krangel, 1987; Minami et al., 1987; Liu et al., 2000). This constitutive cycling depends on the di-leucine-based receptor-sorting motif in the CD3-γ subunit (Minami et al., 1987; Menne et al., 2002). Once the TCR is activated by peptide bound to MHC, anti-TCR antibody or other ligands, it undergoes downmodulation (Cai et al., 1997; Valitutti et al., 1997; Liu et al., 2000). There are at least two distinct pathways for endocytosis of triggered TCR. One is dependent on protein tyrosine kinase activity, leading to TCR ubiquitination (Valitutti et al., 1997; D'Oro et al., 1997; Wang et al., 2001); the other is dependent on protein kinase C (PKC)-mediated activation of the di-leucine motif in the CD3-γ subunit and leads to receptor recycling (Menne et al., 2002; Dietrich 1998). In addition, unengaged TCR are endocytosed (co-modulated) by the PKC/CD3γ-dependent pathway; this might attenuate signaling and/or ensure an internal store of TCR that can be rerouted to the immunological synapse during encounter with an APC (San Jose et al., 2000; Monjas et al., 2004).

In the PKC/CD3γ-dependent pathway of receptor downmodulation, following TCR triggering, PKC is activated and phosphorylates Ser^{126} of CD3γ, which renders the di-leucine motif accessible to binding by AP2 clathrin adaptor protein. AP2 binding to CD3γ leads to TCR endocytosis (Dietrich et al., 1994; 1996; 1997).

The TCR/CD3 downmodulation pathway leading to receptor degradation is intimately linked to TCR signaling, and in particular to the PTK Lck and Fyn. Inhibition or absence of Src family kinases prevents TCR endocytosis (Thuillier et al., 1991; Luton et al., 1994; D'Oro et al., 1997). Phosphorylation events initiated by these kinases lead to recruitment of adaptor molecules, including SLAP and SLAP2, which bring Cbl proteins into close proximity with Src kinases as well as LAT, ZAP70 and TCR-ζ. The Cbl is further integrated into the complex by binding to the central adaptor molecule SLP76. Src kinases in the complex activate Cbl proteins that in turn ubiquitinate ZAP70, Lck and TCR-ζ and target them

for internalization and degradation (Wang et al., 2001).

Crotzer and colleagues offered another regulatory mechanism that links TCR to endocytosis; they suggested that TCR internalization after receptor engagement involves inducible phosphorylation of clathrin heavy chain (CHC; Crotzer et al., 2004). They showed that Lck is the enzyme responsible for this process and that its activity is influenced by the function of active ZAP70.

The half-life of the interaction between pMHC and the TCR modulates the extent to which the TCR is phosphorylated. Lee et al. demonstrated that unphosphorylated or partially phosphorylated TCR (corresponding to the p21 form) is returned to the cell surface after internalization, whereas fully phosphorylated TCR (the p23 form) is degraded and removed from the system after internalization (Lee et al., 2003). These observations added to the ongoing debate regarding the role of cSMAC in TCR downmodulation. By clustering TCR, cSMAC facilitates full phosphorylation by Lck; because only fully phosphorylated receptors are subject to degradation, cSMAC thus both enhances and limits TCR signaling. This regulatory function of cSMAC on TCR activation also takes into account the antigen concentration. At low peptide-MHC concentrations, cSMAC enhances T cell activation by clustering engaged TCR, however at high peptide-MHC concentration, cSMAC can become a site of TCR downmodulation and degradation. In addition, Lee and colleagues demonstrated that AP2 recruitment is critical for the degradation of activated TCR/CD3 complexes, and that T cells deficient in this protein show impaired TCR downmodulation (Lee et al., 2003).

The role of Fas in T cell activation

Evidence to date suggests that, in addition to its proapoptotic effect, Fas/FasL signaling has other important cell or tissue-dependent properties (Strasser et al., 2009). The non apoptotic or costimulatory consequences of Fas engagement have been described for different cell types during liver regeneration, neurite outgrowth, development and functional recovery of the central nervous system, and also proliferation of growth factor-deprived fibroblasts (Peter et al., 2007). Fas affects proliferation, differentiation and migration processes as well as cytokine production in various hematopoietic and non-hematopoietic cell types.

Fas binding might be very relevant in the modulation of TCR/CD3 signaling in primary T cells, and most reports identify Fas as a costimulatory molecule for T cells. Alderson et al. provided the first evidence for this function of Fas showing that the presence of a functional Fas/FasL system enhances proliferation and cytokine production in the activation of human T cells (Alderson et al.,

1993). A later study demonstrated the costimulatory function of Fas in murine naïve T cells, and that Fas costimulation is controlled by NF-κB signaling and caspase activity (Maksimow et al., 2006). It was also shown that Fas might have a role in mature T cell activation, although Fas-mediated T cell proliferation was observed only with a full mitogenic dose of anti-CD3, in contrast to the effect of CD28 costimulation, which is observed even at suboptimal concentrations of anti-CD3 (Chun et al., 2000).

As shown above, several reports demonstrate a potent regulatory function of Fas in T cell activation. The precise molecular mechanism of Fas costimulation, however, had never been elucidated in detail. Understanding of these additional Fas functions was further complicated by reports that identified Fas as a silencer of T cell activation. The authors showed that crosslinking of the Fas antigen on preactivated human T cells inhibits early TCR/CD3 signaling events such as intracellular Ca^{2+} mobilization (Kovacs and Tsokos, 1995). These results were subsequently completed by the same investigators, who showed impaired association of ZAP70 with TCR-ζ in Jurkat cells after Fas crosslinking (Kovacs et al., 1999).

In accordance with these data, others reported that CD95 triggering during priming of naïve human T cells interfered with proximal TCR signaling by inhibiting ZAP70, PLC-γ1 and PKC recruitment into lipid rafts, as well as mutual tyrosine phosphorylation of these proteins. Subsequent Ca^{2+} mobilization and nuclear translocation of NF-κB, NFAT, and AP1 were greatly reduced, resulting in impaired cytokine production and cell proliferation. In addition, these cells were partially non-responsive after secondary T cell stimulation (Strauss et al., 2009). In line with these data, Paulsen et al. reported that high CD95 agonist concentrations block activation of human naïve T cells by inhibiting TCR signaling (Paulsen et al., 2011), although they demonstrated dual Fas modulator function during TCR/CD3 activation of primary human T cells. Fas can have opposite effects depending on the agonist concentration, ranging from complete activation blockade at high doses to a prominent costimulatory role at lower concentrations. T cell activation induced by the phorbol ester PMA (phorbol 12-myristate 13-acetate) and ionophore (ionomycin) was unaltered by Fas binding at either high or at low concentration, indicating that Fas affects TCR signaling upstream or independently of PKC and Ca^{2+} mobilization. Moreover, Fas crosslinking was shown to promote TCR internalization (Paulsen et al., 2011).

These reports describe the impact of Fas engagement on T cell activation. The functional TCR complex was recently observed to have an essential role in Fas mediation of apoptosis (Akizhanov et al., 2010), suggesting that the interaction between Fas and TCR signaling pathways is bidirectional. Using immunoprecipitation assays, Akizhanov et al. showed that Fas interacts directly with the TCR complex

proteins Lck and PLC-γ1, which suggests that the unengaged TCR complex can act as a direct regulator of Fas signaling. The strong dependence of Fas-induced cell death on the presence of active Lck was reported previously by Sharif-Askari et al., who also showed Lck preassociation with the Fas signaling complex in untreated T cells (Sharif-Askari et al., 2007). Direct Fas association with another Src family member, Yes, and with the PI3K p85 subunit was also described in glioblastoma cells (Kleber et al., 2008). Fyn, another T cell-associated tyrosine kinase, was also found to interact physically with Fas in activated lymphocytes (Atkinson et al., 1996). This last report describes the presence of the tyrosine-containing motif YXXL within the CD95 DD sequence, similar to canonical ITAM or ITIM (immunoreceptor tyrosine inhibitory motif) found in the TCR/CD3 complex, opening up the possibility of Fas interaction with SH2-containing proteins.

Considerable effort has been made to understand Fas functions in addition to its known role in apoptosis induction. As shown above, some reports indicated a role for Fas as a potent modulator in T cell activation, although the underlying molecular mechanism has not been explained in detail. Published data on Fas costimulation are inconsistent, since Fas was described as both an enhancer and a silencer of primary T cell activation. In addition, these results define Fas functions mainly in human primary cells. As study of these Fas characteristics in effector/memory T cells has been limited, the overall biological function of Fas signaling remains unknown. In this thesis, we examined the nature of hyperproliferating Fas-deficient T cells and studied their involvement in lupus-like disease in B6/*lpr* mice. We show that reduction of T cell hyperproliferation indeed attenuates disease manifestations in these animals, and identify Fas as a potent negative regulator of proliferation in preactivated T cells. Our data indicate that Fas associates with the TCR complex and affects its internalization. Based on these findings, we propose a mechanism of T cell homeostasis that implicates this alternative to the proapoptotic function of Fas.

As an apoptosis-inducing factor, Fas plays an important role in T cell homeostasis (Watanabe-Fukunaga et al., 1992). This healthy state is achieved through a balance in cell proliferation, survival and apoptosis. When one of these processes is impaired, the balance is destabilized. This can lead to accumulation of autoreactive and activated T cells and, ultimately, to autoimmune disease development; this is the case of Fas-deficient B6/*lpr* mice. These mice show a lymphoproliferative lupus-like syndrome with severe consequences, including kidney failure and death (Walker et al., 2002). Although this autoimmune disease is extensively studied, its etiology remains elusive. *In vitro* death defects, which seem to be the most logical explanation, do not justify the B6/*lpr* phenotype, as the absence of other proteins involved in apoptosis, such as FADD and caspase-8, do not lead to this autoimmune phenotype (Newton et al., 1998; Newton et al., 2001; Zhang et al., 1998; Chun et al., 2002; Smith et al., 1996). Hyperproliferation of T cells from B6/*lpr* mice is also poorly understood and reports suggest that this hyperproliferation is essential for lymphadenopathy development (Balomenos et al., 1997; Fortner et al., 2005; Katagiri et al., 1988). Thus, we considered that alternative yet unknown Fas properties might account for the B6/*lpr* phenotype, in addition to its pro-apoptotic function. With these observations in mind, we studied the role of Fas in T cell homeostasis, which appears to be more complex than simply apoptosis induction. Furthermore, we analyzed the effect of the cell cycle inhibitor p21 overexpression on the B6/*lpr* hyperproliferation.

We established the following objectives:

1. To analyze a possible proliferative advantage of Fas- and FasL-deficient T cells over control T cells and their NF-κB activation status
2. To study the importance of the Fas-FasL interaction and the molecular mechanism underlying the Fas inhibitory effect on secondary T cell proliferation
3. To determine where in the activation/proliferation signaling Fas exhibits its inhibitory role
4. To identify the relationship of Fas with TCR in T cell activation and proliferation
5. To attempt to inhibit B6/*lpr* hyperproliferation
6. To analyze the *in vivo* effect of p21 overexpression in B6/*lpr* mice

Mice

Control C57BL/6 mice were from Harlan Interfauna Ibérica; C57BL/6-*lpr* (B6.MRL-*Tnfrsf6lpr*), C57BL/6-*gld,* MRL-mp and MRL-*lpr* were from Jackson Laboratories. C57BL/6-p21tg mice were a kind gift from Arun Fotedar (Sidney Kimmel Cancer Center, San Diego, CA). C57BL/6-*lpr*-p21tg mice were generated from C57BL/6-*lpr* and C57BL/6-p21tg mice. MRL-*lpr*-p21tg mice were generated by backcrossing C57BL/6-p21tg with MRL-*lpr* mice over more than nine generations. In both C57BL/6-*lpr*-p21tg and MRL-*lpr*-p21tg, transgene expression was restricted to T cells by the proximal Lck promoter (Fotedar et al., 1999). All mice were maintained at the CNB animal facility. For most experiments, we used mice of less than eight weeks of age to study molecular and cellular events that precede autoimmune disease development. All animal experiments were performed in accordance with European regulations, and were approved by the CNB Bioethics Committee.

T cell purification and culture

Isolation of CD4+ T cells

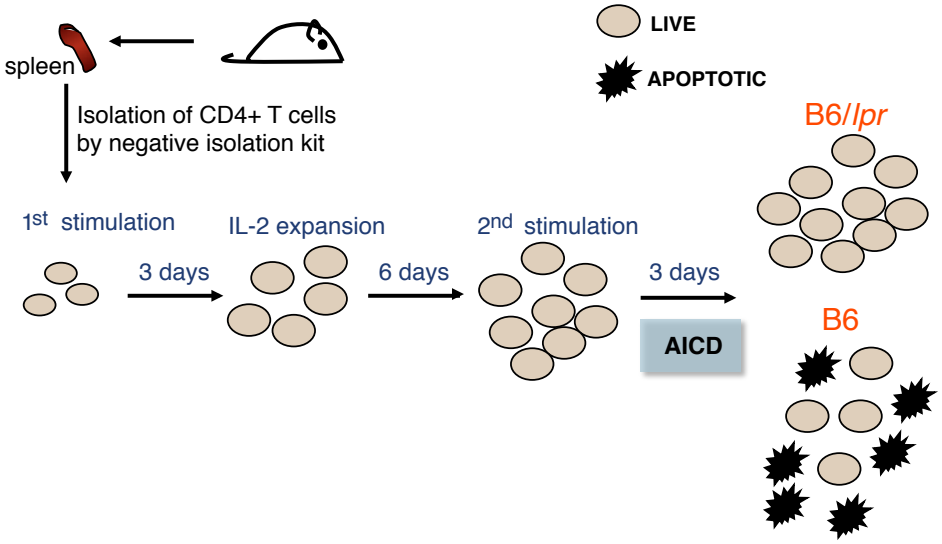
Mouse CD4+ T cells were obtained from a single-cell spleen suspension by negative selection (Mouse CD4 Negative Isolation Kit, Dynal Biotech) with magnetic beads coated with antibodies Tib 105, 3F12, RA3-6B2, as well as PK136 and CD11b, to bind CD8+ cells, B cells, NK cells and macrophages, respectively. After 30 min incubation, a magnetic field was applied and CD4+ cells were separated. Purity was confirmed by flow cytometry and was routinely >90%.

Cell culture

CD4+ T cells were cultured in RPMI-1640 medium (Gibco) supplemented with 100 U/ml penicillin-streptomycin, 10 mM HEPES pH 7.4, 50 mM β-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (all from Gibco) and 10% FCS (fetal calf serum; Harlan Bioproducts).

Repeated cell stimulation protocol

Activation-induced cell death (AICD) was induced using repeated cell stimulation protocols. Purified CD4+ T cells (10^6 cells/ml) were stimulated *in vitro* with 3 μg/ml concanavalin A (ConA; Sigma), and the culture was supplemented with 20 ng/ml human recombinant interleukin-2 (rIL-2; PeproTech) for 3 days (Scheme 5). In some experiments, cells were stimulated with plate-bound anti-CD3 (0.5 μg/ml; PharMingen) in 0.1 M Tris-HCl and soluble anti-CD28 (1 μg/ml; PharMingen), or with ionomycin (0.5 μg/ml; Sigma) and PMA (20 ng/ml; Calbiochem). Cells were then washed and resuspended in fresh culture medium supplemented with 20 ng/ml IL-2 for 6 days. During this time,



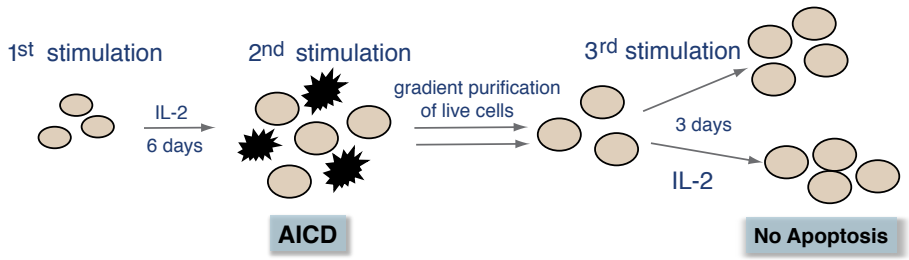
Scheme 5. *In vitro* AICD protocol
After extraction of CD4+ T cells from murine spleen using a negative isolation kit, cells were ConA-stimulated (3 days), IL-2-expanded (6 days) and ConA-restimulated (3 days).

medium was changed once to avoid nutrient and IL-2 depletion. Cell concentration during IL-2 expansion was 0.5 x 10^6 cells/ml. Cells (10^6/ml) were then restimulated with 3 μg/ml ConA and 20 ng/ml IL-2 for 3 days.

Some cells were cultured with the cell death inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethlketone (zVAD; Bachem), used at the indicated concentrations.

For the apoptosis-free conditions system, after 48 h of secondary ConA stimulation, cells were washed and live cells separated on a Ficoll gradient (800 x g, 20 min; Lympholyte, Cedarlane Laboratories). Propidium iodide staining (PI; DNA-Prep reagent Kit, Beckman Coulter) and cell cycle analysis ensured that after Ficoll treatment, >80% of cells were alive and cycling. After separation, control and B6/*lpr* T cells were plated at the same concentration and restimulated for 72 h. Alternatively, after separation cells were expanded in IL-2-containing medium (Scheme 6).

In the repeated stimulation experiments with B6/*gld* T cells, we used 150 ng/



Scheme 6. Apoptosis-free conditions protocols. After 48 h of secondary stimulation, live CD4+ cells were gradient-separated from apoptotic cells and were either ConA-restimulated (3rd stimulation) or cultured with IL-2. In these conditions, apoptosis does not occur.

ml recombinant FasL and 50 ng/ml crosslinking enhancer (both from Alexis Biochemicals).

Flow cytometry (fluorescence activated cell sorting, FACS)

All staining was performed by incubation of cells in 50 μl of appropriate antibody mixture (depending on experimental context) for 20 min at 4ºC, in the dark. Antibodies were resuspended in PBS staining buffer (1x PBS, 5% FCS, 1% BSA, 5 mM EDTA). After incubation, cells were washed twice with PBS staining buffer and analyzed on an LSR cytometer (Becton Dickinson). Experiments were analyzed using FlowJo software (Tree Star). Freshly isolated $CD4^+$ T cells were stained to determine memory phenotype with anti-CD4-PE (Beckman-Coulter), -CD8-PEcy7 (Biolegend), -CD44-FITC (Beckman) and -CD62L-APC (Beckman-Coulter) antibodies. For T cell activation analysis, cells were stained with anti-CD4-FITC, -CD25-PE (both from PharMingen) and -CD69-Pecy7 (eBioscience). For double negative population analysis, cells were stained with anti-CD4-PE, -CD8-PEcy7, -B220-APC (Beckman-Coulter), -TCR-FITC (PharMingen) and -Thy1.2-APC (PharMingen) antibodies.

BrdU was detected with an anti-BrdU-FITC antibody (Becton Dickinson) in combination with anti-CD4-Pecy7 (eBioscience), -CD8-SPRD (Beckman-Coulter), -CD44-biot (PharMingen), Av-SPRD (Beckman-Coulter), -CD62-PE (Southern) and -TCR-PE antibodies.

Proliferation assays

[^{3}H]thymidine incorporation

T cell proliferation was measured by culturing cells in 96-well plates for indicated times and quantifying [^{3}H] thymidine uptake (1 mCi/150 ml; Amersham Bioscience) in the last 16 h of culture. Incorporated radioactivity was measured using a beta-plate counter (b1205 Wallac, Perkin Elmer) after transferring cells onto fiber filters (Wallac) with a semi-automatic cell harvester.

CFSE fluorescence dilution assay

Cells were washed with PBS and resuspended in PBS + 0.1% BSA at a final concentration of 2 x 10^6 cells/ml and incubated with 2.5 μM of the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) for 15 min at 37ºC. The reaction was terminated by incubating cells on ice. Cells were washed three times with cold RPMI-1640 medium and stimulated according to the requirements of each experiment. CFSE fluorescence decay at the indicated time points was measured by flow cytometry, gating live PI-negative cells.

Cell cycle analysis

For cell cycle analysis, cells were washed with PBS at the times indicated, resuspended in 50 μl of detergent (DNA-Prep Reagent Kit, Beckman Coulter) and 10 mg/ml PI was added (DNA-Prep reagent Kit; 30 min, 37ºC) before flow cytometry analysis.

Ki-67 proliferation marker staining

After surface marker staining, cells were washed with PBS and fixed and permeabilized with 100 µl Cytofix/Cytoperm (BD Biosciences; 20 min, 4ºC). After incubation, cells were washed once with Perm/Wash buffer (BD Biosciences) and blocked with 150 µl Perm/Wash buffer + 1% BSA (30 min, room temperature). Anti-Ki-67 antibody (1/100, Abcam) was added; the permeabilized cells were incubated (1 h, 4°C) and washed twice in Perm/Wash before flow cytometry analysis.

Trypan blue exclusion test of cell viability

The number of live cells as determined by counting Trypan blue-stained cells was also used to measure proliferation ratio.

In vivo BrdU administration

Mice were given bromodeoxyuridine (BrdU, 0.8 mg/ml; Sigma) in drinking water, freshly prepared every 2 days for a 9-day period, and BrdU incorporation was determined in lymph node T cells (Balomenos et al., 1997). Cells were stained with appropriate combinations of labeled anti-CD4, -CD8 and -CD44, -CD62L, -TCR, -Thy1.2 and -B220 antibodies and anti-BrdU antibody (for antibody sources, see flow cytometry section).

Apoptosis assay

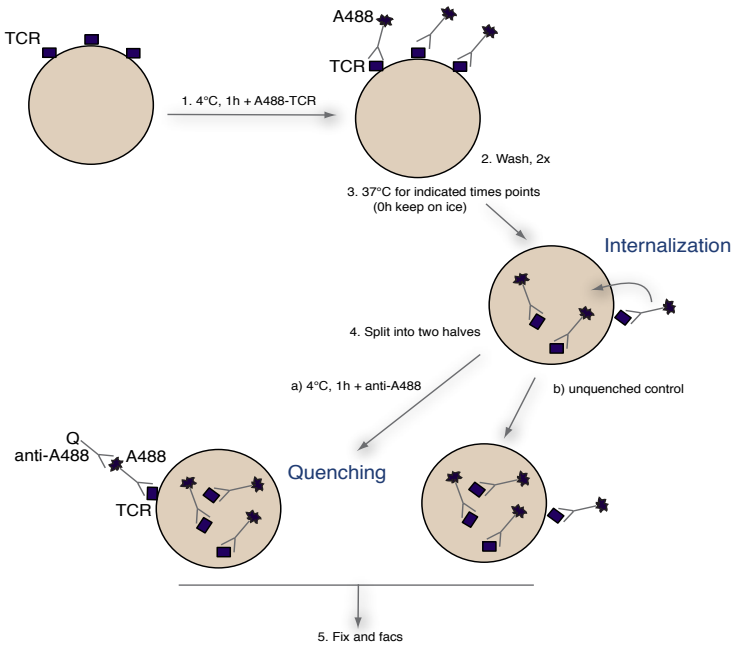
For annexin-V and PI double staining, cultured cells at the indicated time were stained using an anti-annexin-V antibody kit (PharMingen, 5 µl/100 µl; 15 min, 4ºC). To distinguish live from apoptotic and necrotic cells, 10 µl of 50 µg/ml PI stock solution was added before flow cytometry analysis.

Sorting of DN and memory T cells

Mouse CD4+ T cells were isolated from spleen using the Negative Isolation Kit and stained with anti-TCR-FITC, -CD4-PE and -CD8-SPRD for the DN T cell population, and with anti-CD4-PE, -CD44-FITC and -CD62L-APC for the memory T cell population. Cell populations of interest were sorted on an Epics Altra sorter (Beckman-Coulter).

Intracellular cytokine staining

For intracellular cytokine staining, cells were isolated from spleen, then plated (5 x 10^6 cells/ml) and stimulated (1 h, 37°C) in culture medium containing PMA (50 ng/ml), ionomycin (2 µg/ml). Brefeldin A (GolgiPlug, 10 µl/ml; BD Pharmingen) was added and cells were further incubated (3 h, 37°C). After surface marker staining (CD4, CD8, CD44, CD62L, Thy1), cells were washed with PBS, fixed, and permeabilized with 100 µl Cytofix/Cytoperm (20 min, 4ºC). Cells were then washed once with Perm/Wash buffer and blocked with 150 µl Perm/Wash buffer + 1% BSA (30 min, room temperature). Anti-IL-17-PE (1/100, PharMingen) and anti-IFNγ-PE (1/100, PharMingen) antibodies were added; the permeabilized cells were incubated (20



Scheme 7.
Internalization assay
Surface TCR was stained with Alexa488-conjugated antibody. Cells were pulsed at 37°C to internalize receptor and were then divided into two groups; one group was fixed and the other incubated with quenching antibody prior to fixation and FACS analysis.

min, 4°C) and washed twice in Perm/Wash before FACS analysis.

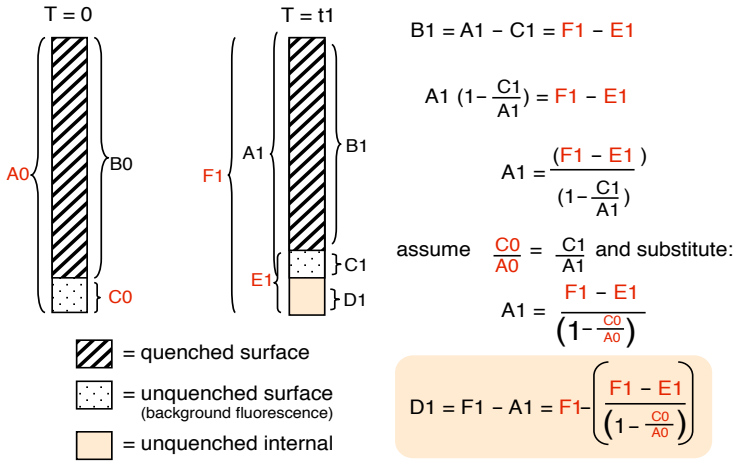
Internalization and recycling assays using cell surface fluorescence quenching

For TCR internalization, cells following the first or second stimulation or during the IL-2 expansion phase were incubated (45 min, on ice) with 2 μg/ml Alexa488-conjugated anti-TCRβ (Invitrogen), which binds the TCR stably. Cells were washed three times in medium and pulsed for indicated times at 37°C. Internalization was terminated by placing samples on ice. Cells were divided into two groups; one was fixed with 2% formaldehyde and the other incubated (1 h, on ice) with 25 μg/ml rabbit anti-Alexa488 quenching antibody (Molecular Probes) prior to fixing and flow cytometry analysis (Scheme 7).

Mean fluorescence intensity was quantified for 6 x 10^3 viable cells by flow cytometry, and internalized fluorescence was calculated from quenched and non-quenched samples, as described (Austin et al., 2004) (Scheme 8). Surface antibody and TCR endocytic rate constants were determined by generating internalization plots.

In some experiments, cells at the end of the IL-2 expansion phase were ConA restimulated (1 h) to trigger internalization. Cells were then incubated (37°C) with Alexa488-transferrin (Molecular Probes) for indicated times. Cells were washed and divided into two groups; one group was fixed directly and the other, incubated with quenching antibody prior to fixing and FACS analysis. Data are representative of two experiments.

For TCR recycling, cells were pre-incubated (15 min, 37°C) with 2 μg/ml Alexa488-anti-TCRβ antibody. Cells were chilled rapidly, washed twice in cold medium and surface-quenched (15 min, on ice) with 25 μg/ml anti-Alexa488 antibody. Cells were then warmed to 37°C (chased) in the continuous presence of



Scheme 8. Calculation of internalized receptor amount from raw FACS data. The amount of internal signal (D1) is calculated at different time points. Surface fluorescence (A1), which consists of quenched fluorescence (B1) and nonquenchable background fluorescence (C1), is subtracted from total unquenched fluorescence (F1) at each time point. Background fluorescence as well as the ratio of quenched and total unquenched fluorescence at time 0 (1-C0/A0; quenching efficiency) are assumed to be the same at all time points.

the quenching agents for the indicated intervals, chilled rapidly, fixed and analyzed by flow cytometry (Scheme 9).

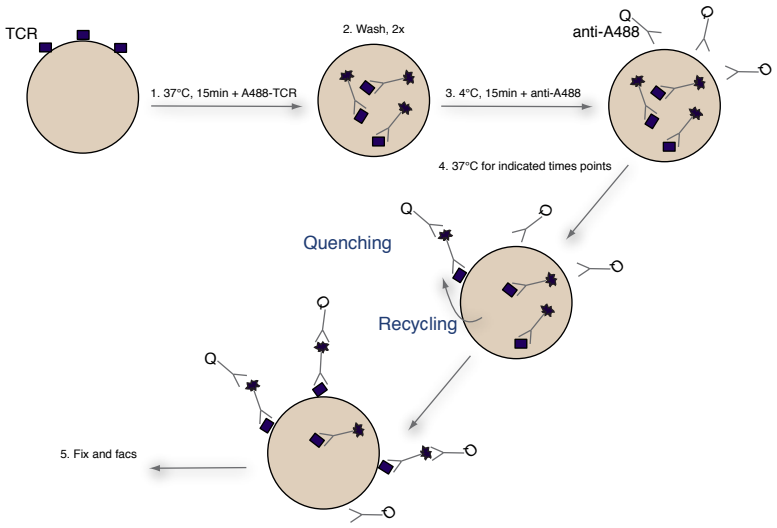
The percentage of internalized and recycled fluorescence (pulsed) remaining at each chase time point was calculated as the difference between pulsed and unpulsed cell fluorescence, normalized to this value at chase time = 0, as described (Austin et al., 2004).

Calcium (Ca^{2+}) mobilization assay

Cells were washed with PBS and resuspended in cold Tyrode's solution (Sigma), after which 20 μl 10 mM Fluo-2 (AM) (Teflabs) was added and incubated (15 min, room temperature). Cells were centrifuged, resuspended in 5 ml Tyrode's solution and incubated (30 min, room temperature). The baseline was established by collecting events for 100 s on an LSRFortessa cell analyzer (BD Biosciences). These cells were then stimulated (5 μg/ml CD3 and 1 μg/ml CD28, or 0.5 μg/ml ionomycin and 20 ng/ml PMA) and further events collected on the same equipment.

Western blot

Cells were lysed in 0.2% NP-40 lysis buffer supplemented with Phosphatase Inhibitor Cocktail (PhosStop) and Complete Protease Inhibitor Cocktail (both from Roche). Protein concentration was determined using the Bradford method. Lysates (40 μg) of freshly isolated or cultured cells were resolved on 12% acrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5% low fat milk (or BSA) in 0.05% Tween 20, membranes were incubated with appropriate primary antibodies. After incubation with appropriate peroxidase-conjugated secondary antibodies (all



Scheme 9. Recycling assay. Cells were stained with Alexa488-anti-TCRβ antibody at 37°C to internalize the labeled receptor. Cells were then incubated on ice with quenching anti-Alexa488 antibody and warmed to 37°C in the continuous presence of the quenching agent. Recycling of Alexa488-labeled TCR induced quenching of surface fluorescence.

from Dako), proteins of interest were detected by chemiluminescence (ESL, Amersham) according to manufacturer's indications. Primary antibodies were from Cell Signaling (anti-pSrk, -pZAP70, -pAkt, -pPKCα, -pPKCθ, -pLAT, -pIκBα, -pERK, -Lck, -ZAP70, -ERK, -MALT1, -Bcl10, -acetyl coA), Santa Cruz Biotechnology (anti-p21, -CDK2, -TCRγ, -Fas, -TRAF6, -LAT), Millipore (anti-phosphotyrosine), Alexis (anti-caspase-8), MBL (anti-cFLIP, -FADD), ProSci (anti-CARMA1) and Sigma (anti-β-actin).

Phosphotyrosine immunoprecipitation

Untreated and ConA-restimulated (15 min) B6 and B6/*lpr* T cells were lysed in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1mg/ml aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 1 mM NaF). Protein (250 μg) was incubated (overnight, 4°C) with 20 μl 4G10 Platinum Anti-Phosphotyrosine agarose conjugate (50% slurry; Millipore); beads were then separated from supernatant by microcentrifugation (4010 x g, 4 min) and washed three times with PBS. Proteins were dissociated from beads with SDS-PAGE sample buffer, resolved on 12% acrylamide gels and transferred to a nitrocellulose membrane. Immunoblotting was performed with a protein-specific antibody.

Biotin-VAD-fmk caspase precipitation assay

T cells were incubated with 10 mM biotin-VAD-fmk (bVAD; MP Biomedicals; 30 min, 37ºC). Cells were lysed with 0.2% NP-40 lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl) containing an additional 20 mM bVAD (20 min, on ice). After protein quantification, cell lysates (400-600 μg protein) were pre-cleared by incubation (2 h, 4°C) with Sepharose 6B beads (30 ml; Pierce), followed by incubation (overnight, 4°C) with 40 ml immobilized streptavidin beads. Beads were washed 5 times with lysis buffer without protease inhibitor cocktail and boiled with loading buffer (5 min). Proteins were separated on 12% polyacrylamide gels and precipitated caspase-8 was detected by western blot.

Caspase-8 colorimetric activity assay

Caspase-8 activity was determined by measuring fluorescence originating from a cleaved tetrapeptide (IETD) substrate linked to a fluorogenic group (pNA) using the FLICE/Caspase-8 Colorimetric Assay Kit (BV-K113, 3 MBL). Viable cells were lysed and treated according to the manufacturer's protocol. Caspase-8 recognizes the IETD sequence, and cleavage of the tetrapeptide substrate by active caspase-8 in cell lysates releases the pNA group; light emission is quantified at 400-405 nm by spectrofluorometry to determine the increase in caspase-8 activity.

RNAi transfection of T cells

Small interfering RNA (RNAi) specific for caspase-8 bearing two different targeting sequences (sc-37226; Santa Cruz) and the non-specific control (sc-37007; Santa Cruz) were used to transfect *lpr* T cells at the IL-2 expansion stage, using the Amaxa Nucleofector system for mouse lymphocytes (VPA-1002). Cells were then incubated overnight and cultured with IL-2 for 2 days prior to secondary ConA stimulation.

Confocal analysis

Cells were cultured overnight on collagen-I-covered chamber slides (Lab-Tek) and left untreated or stimulated with 3 μg/ml ConA, 20 ng/ml IL-2 and 30 μM zVAD (1 h, 37°C). Cells were then placed at 4°C to terminate stimulation, fixed, and permabilized with Cytofix/Cytoperm solution (BD Biosciences; 30 min, 4°C). Cells were washed twice with Perm/Wash solution (BD Biosciences) and incubated with blocking solution (PBS + 1% BSA + 10% goat serum; 45 min, room temperature) in a humid chamber. After washing with Perm/Wash, anti-TCRg and -Fas (A-20) antibodies (both from Santa Cruz Biotechnology) were added and cells incubated (45 min, room temperature) in a humid chamber. For some experiments, cells were incubated with anti-caspase-8 antibody (Alexis Biochemicals). After 1 h, cells were washed three times with PBS to remove unbound primary antibody; anti-goat IgG (Santa Cruz Biotechnol-

ogy) and -rabbit IgG (Molecular Probes) secondary antibodies were added (1 h, room temperature). After mounting cells with DAPI-containing mounting medium (Vectashield; Vector Laboratories), fluorescence was documented on a confocal microscope (Olympus).

NF-κB activation assays

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained by lysing cells in Buffer A (see table below; 5 min, 4°C). After centrifugation (4010 x g, 4 min), supernatant (cytosolic extract) was separated from the pellet (containing nuclei), which was washed with Buffer B (see table). After centrifugation (4010 x g, 4 min), supernatant was removed and the pellets incubated (30 min, 4°C) with Buffer C (see table). Subsequent centrifugation (4010 x g, 15 min) completed nuclear extraction. The nuclear extracts (10 μg) were incubated in binding buffer (0.45 μg/μl BSA, 20 mM Hepes, 1 mM EDTA, 20% glycerol, 1 M DTT, 0.5 mM PMSF, 1 M MgCl, 0.5 M EDTA pH 7, 2 M NaCl, 100 mM Tris-HCl pH 7.5, poly(dI-dC) with 1 μl [γ-^{32}P]-ATP-labeled probe containing the NF-κB consensus sequence (sense: 5´-AGTTGAGGGGACTTTCCCAGGC-3´, antisense 3-TCAACTCCCCT-GAAAGGGTCCG-5´, Promega). The labeling reaction was catalyzed by T4-polynucleotide kinase and incubated in 10x kinase buffer (30 min, 37°C). Nuclear extracts were incubated (20 min, room temperature) with radiolabeled probe and resolved on 5% acrylamide gels. As a loading control, probe containing the NF-Y consensus sequence was used. Gels were dried and exposed to X-ray film (Kodak BioMax MR) for up to 3 days at -80°C. After development of the autoradiographs, band intensity of radiolabeled NF-κB-DNA complexes was used to measure NF-κB activation.

Table 1. Lysis buffer composition

	Buffer A (100 ml)	Buffer B (25 ml)	Buffer C (20 ml)
Hepes (1 M)	1000 µl	250 µl	200 µl
NaCl (5 M)	1000 µl	250 µl	1400 µl
Saccharose (100 mM)	500 µl	-	-
EDTA (500 mM)	200 µl	5 µl	4 µl
Spermidine (500 mM)	100 µl	-	20 µl
Spermine (500 mM)	30 µl	-	6 µl
Triton X-100 (100%)	500 µl	-	-
Glycerol	-	6.25 ml	5 ml
ddH2O	97 ml	18.25 ml	13.4 ml

TransAM ELISA-based kit

This kit contains 96-well plates to which an oligonucleotide containing an NF-κB consensus binding site has been immobilized. Activated NF-κB homo- and heterodimers in nuclear extracts (obtained according to manufacturer's protocol with reagents provided) bind specifically to the oligonucleotide. The activated NF-κB subunit bound to the oligonucleotide is detected with an anti-NF-κB p65 antibody. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout easily quantified by spectrophotometry.

IN Cell Analyzer 2000 cell imaging system

The percentage of cells positive for NF-κB translocation was defined with an IN Cell Analyzer 2000 (GE Healthcare) high-content analysis system. The software localizes the nuclei based on AAD nuclear stain (Invitrogen) and defines the nuclear space. The algorithm then defines a collar space around the nuclei. Signal intensity for NF-κB (NF-κB, p65, Santa Cruz) is then measured in the nuclei and in the peri-nuclear space, as a surrogate for cell staining. NF-κB activation status is defined by its nuclear versus cytoplasmic localization. Data are expressed as the ratio between nuclear and cytoplasm staining intensity.

Detection of anti-DNA antibodies

The relative levels of anti-DNA antibodies in serum were detected by enzyme-linked immunosorbent assay (ELISA). The 96-well plates were first coated with calf thymus DNA (2.5 µg/ml; Sigma Aldrich). IgG was detected with peroxidase-conjugated anti-mouse antibody (Jackson ImmunoResearch). Relative absorbance levels were measured at 492 nm in a Labsystems Multiskan Plus Plate Reader.

Kidney cryosections and immunofluorescence analysis

Kidneys were isolated, embedded in inclusion solution (Jung Tissue Freezing medium, Leica Microsystems) and frozen individually in liquid nitrogen. Cryosections (7 μm) were prepared in a cryostat (Leica CM1900), placed on Fischer slides (Fisherbrand Superfrost/Plus) and allowed to dry (2 h, room temperature). Sections were then treated with 100% acetone (10 min, 4°C), dried (30 min, room temperature) and blocked with PBS + 2% BSA + 10% goat serum (45 min, room temperature). After washings (3 times in PBS), sections were incubated (1 h, room temperature) with combinations of anti-IgG-FITC, -CD4-PE (both from PharMingen), and -F4/80-FITC primary antibodies (Serotec). After washing (3 times in PBS) and mounting with mounting medium (Vectashield, Vector Laboratories), fluorescence was documented on a fluorescence microscope (Leica).

Glomerulonephritis evaluation

Glomerulonephritis grade was evaluated on kidney sections according to the Berden scale (Berden et al., 1983): (0) no glomerular lesions, (1) minimal thickening of the mesangium, (2) lesions with noticeable increases in mesangial and glomerular cellularity, (3) lesions characterized by the preceding conditions with superimposed inflammatory exudates and capsular adhesions, and (4) the glomerular architecture was obliterated in >70% of glomeruli, and tubular cast formation was extensive. Results were evaluated by Dr. Juana Flores, Animal Biology Department, School of Veterinary Medicine (Universidad Complutense de Madrid).

Statistical analysis

Student's *t* test was used for all statistical analyses; p values <0.05 were considered significant.

Hyperactivation of the immune system in B6/*lpr* mice

B6/*lpr* mice show Fas deficiency that leads to defective apoptosis signaling and an autoimmune phenotype, characterized by development of lymphadenopathy and splenomegaly. These processes originate from the accumulation of double negative (DN) T cells (TCRab+CD4-CD8-B220+), which are otherwise rare components of the peripheral T cell repertoire. The accumulation of CD4+ memory T cells (CD44high/CD62Llow) and of IgG+ B cells was also detected in Fas deficient mice.

We observed that CD4+ T cells in these animals showed an activated phenotype, as they overexpressed the activation markers CD25 and CD69 (Fig. 1A). In addition, CD4+ memory T cells (Fig. 1B) and DN T cells (Fig. 1C) were characterized by higher *in vivo* production of the proinflammatory cytokines IL-17 and IFNγ. In concordance with reports

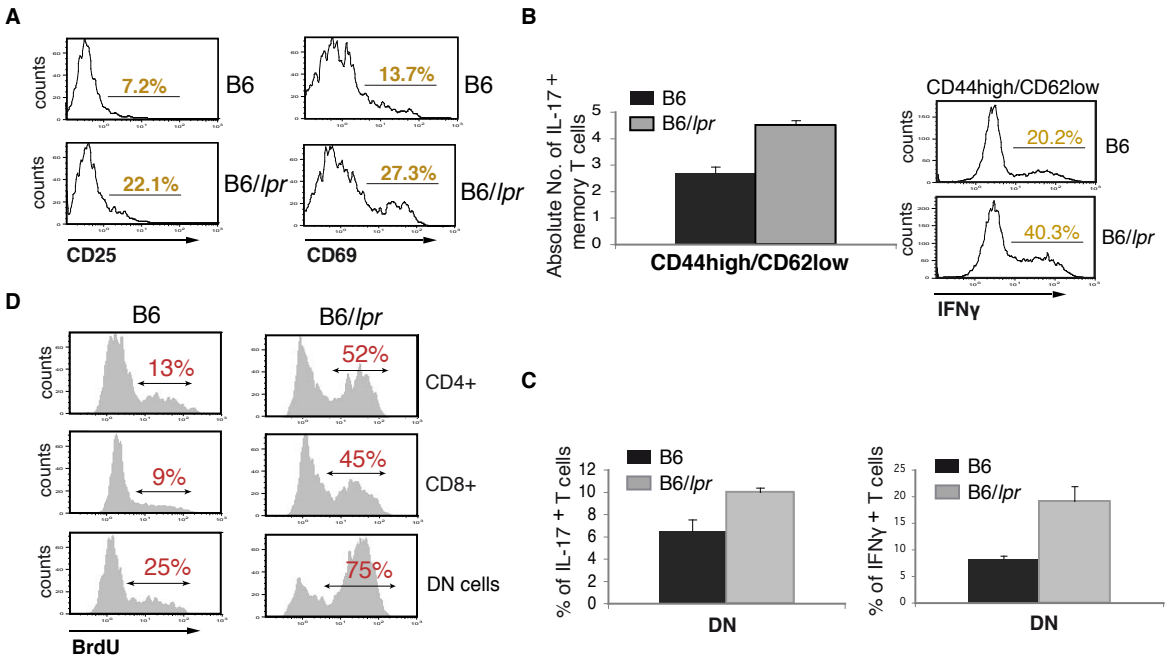


Figure 1. ***In vivo*** **hyperproliferation and hyperactivation of the B6/*lpr* mouse immune system**. **A.** Cell surface expression of activation markers CD25 (left) and CD69 (right) in spleen-derived CD4+ T cells from 2-month-old control and B6/*lpr* mice. Representative histograms are shown (n = 3). **B, C.** Intracellular staining for IL-17 and IFNγ production by memory (CD44highCD62Llow) (B) and DN (Thy1+CD4-CD8-) T cells (C). Single cell suspensions from B6 and B6/*lpr* mouse spleens were prepared, plated (5×10^6 cells/ml), and stimulated with PMA/calcium ionophore and brefeldin A for 4 h. Cells were then collected, fixed and permeabilized, surface markers stained, and cytokine production was measured by intracellular cytokine staining. Values show mean ± SD (n = 4). **D.** CD4+, CD8+ and DN T cells from B6/*lpr* mice showed higher *in vivo* BrdU incorporation than B6 control mice. Two-month-old mice were given BrdU in drinking water for 9 days, after which they were sacrificed and cell suspensions prepared from cervical lymph nodes for staining and FACS analysis. Values are mean ± SD (n = 4).

showing T cell hyperproliferation in B6/*lpr* mice (Balomenos et al., 1997; Fortner et al., 2005), we also observed, using a BrdU incorporation assay, that CD4+, CD8+ and DN T cells in these mice hyperproliferated *in vivo* compared to control T cells (Fig. 1D). This hyperactivation state leads to an autoimmune phenotype with elevated production of anti-DNA antibodies, with deposits of immune complexes in the kidneys and mild glomerulonephritis, finally leading to kidney damage.

Overall, autoimmune disease in B6/*lpr* mice is the result of severe deregulation of immune homeostasis. Since apoptosis defects due to the Fas deficiency cannot explain lymphadenopathy development, hyperactivation or hyperproliferation of T cells from these animals (see Introduction), the aim of this thesis is to show that, in addition to the apoptosis induction, Fas has other functions in immune homesostasis, which are associated with control of T cell activation and proliferation.

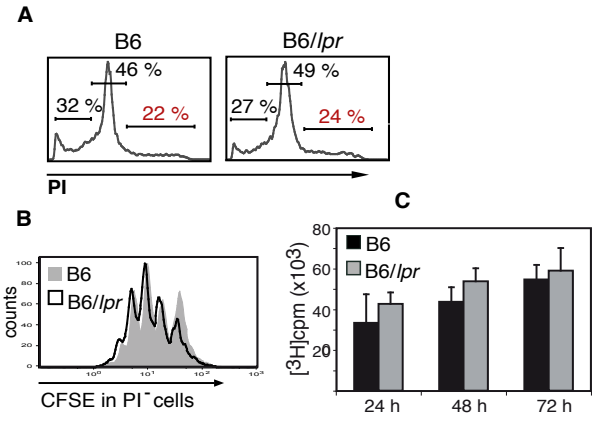


Figure 2. Similar proliferation ratio by B6 and B6/*lpr* T cells after primary stimulation. **A.** Purified CD4+ T cells from B6 and B6/*lpr* mice were ConA-stimulated and PI-stained 24 h later for cell cycle analysis. The proliferation ratio was similar for B6 and B6/*lpr* CD4+ T cells. Representative histograms are shown (n >7). **B.** CFSE dilution rate of gated PI-negative T cells following primary ConA stimulation (72 h). Representative histograms are shown (n = 3). **C.** Similar proliferation of B6 and B6/*lpr* T cells after primary ConA stimulation, detected by [^{3}H]thymidine uptake at 24, 48 and 72 h after first challenge. Values show mean ± SD (n = 4).

Hyperproliferation and hyperactivation of Fas-deficient T cells after repeated stimulation

In the process of an *in vivo* immune response, the first encounter of T cells with antigen is accompanied by a rapid clonal proliferation, followed by a decline in cell number and achievement of a balanced state. Cells that survive form a pool of memory T cells. The second encounter with antigen by circulating T cells is thought to induce apoptosis *via* Fas interaction with FasL. Deregulation of T cell proliferative response or apoptosis can have severe consequences in the immune system homeostasis.

We found that T cells from B6/*lpr* mice hyperproliferate *in vivo.* We therefore studied whether Fas is directly implicated in T cell proliferation control in an apoptosis independent manner. To analyze the proliferative response of B6/*lpr* CD4+ T cells, we used a classical protocol for

activation-induced cell death (AICD), in which naïve T cells are antigen stimulated, expanded with interleukin-2 (IL-2) and afterwards restimulated through their TCR (T cell receptor). This protocol mimics the *in vivo* situation when autoreactive T cells are exposed to the continuous presence of specific autoantigens. To simulate repeated T cell challenge in *in vitro* conditions, we stimulated B6/*lpr* CD4+ T cells repeatedly with concanavalin A (ConA), separated by stages of IL-2 expansion (Scheme 1, Materials and Methods). B6/*lpr* and B6 T cells responded similarly after primary ConA treatment, as determined by cell cycle analysis, CFSE dilution analysis and [^{3}H]thymidine incorporation (Fig. 2A-C). Following the second challenge, B6/*lpr* T cell proliferation appeared to be higher than that of control cells, which underwent extensive apoptosis as detected by cell cycle analysis (Fig. 3A, top). This increased B6/*lpr* T cell pro-

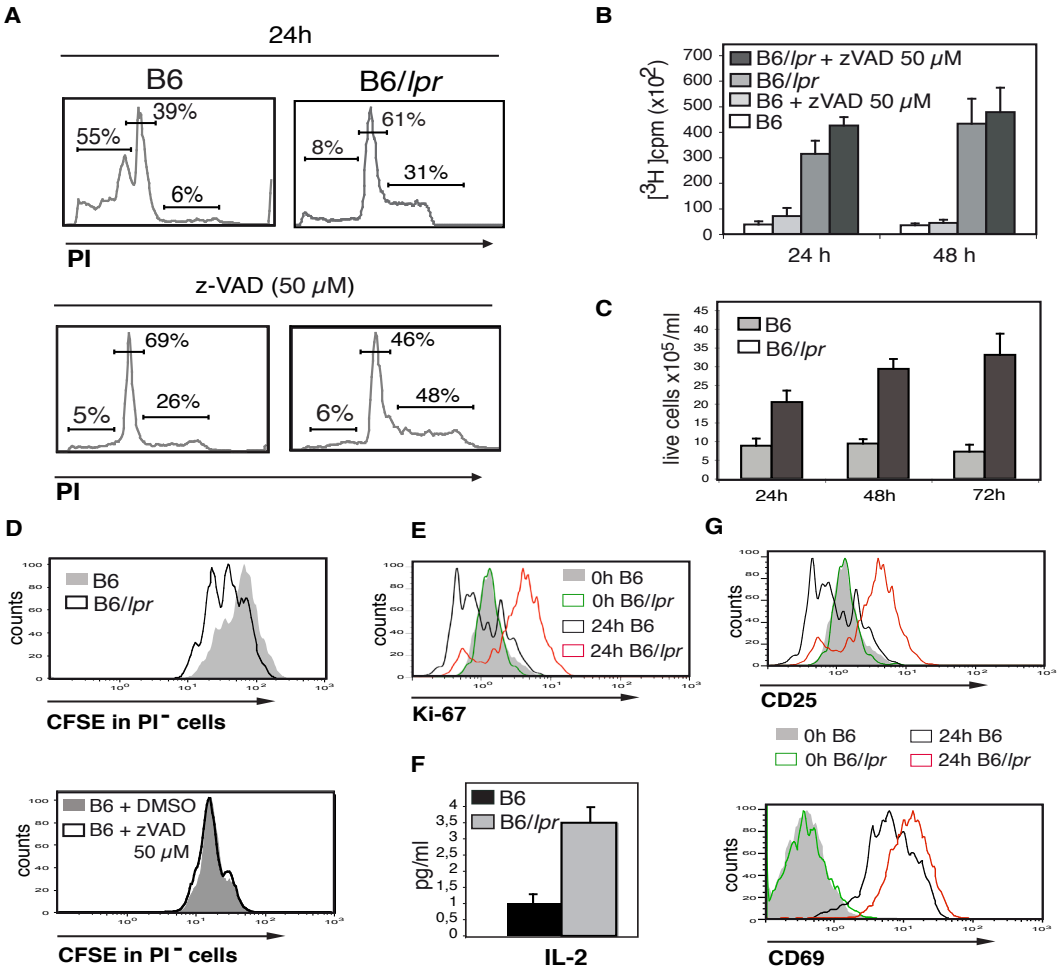


Figure 3. B6/*lpr* T cells hyperproliferate after secondary stimulation. **A.** Purified CD4+ T cells from B6 and B6/*lpr* mice were ConA-stimulated and received a secondary ConA challenge after an IL-2 expansion phase. The proliferation of B6/*lpr* T cells at 24 h post-restimulation was notably higher than that of control T cells, which underwent apoptosis, as measured by cell cycle analysis after PI staining (top). Addition of zVAD (50 μM) efficiently inhibited apoptosis of B6 T cells, although proliferation of B6/*lpr* T cells remained elevated (bottom). Representative data are shown (n = 7). **B.** [^{3}H]thymidine uptake 24 h and 48 h

after secondary ConA challenge. Values show mean ± SD, (n = 6, p<0.0001). **C.** Increased generation of B6/*lpr* T cells after secondary stimulation compared to B6 T cells, determined by dye exclusion based on trypan blue staining. Values show mean ± SD (n = 4; p<0.0005). **D.** CFSE dilution rate of gated PI-negative cells shows increased B6/*lpr* T cell proliferation after secondary stimulation compared to B6 T cells (top). zVAD addition did not affect B6 T cell proliferation (bottom). Representative histograms are shown (n = 4). **E.** Upregulation of the Ki-67 proliferation marker on B6/*lpr* T cells at 24 h after secondary ConA stimulation, as determined by flow cytometry. A representative experiment is shown (n = 3). **F.** High IL-2 levels detected in culture supernatant from B6/*lpr* T cells at 24 h post-restimulation. Values show mean ± SD (n = 4). **G.** Elevated surface expression of CD25 and CD69 activation markers on B6/*lpr* T cells at 24 h post-restimulation, as compared to B6 T cells. Representative histograms are shown (n = 4).

liferation ratio could nonetheless be due to accumulation of live apoptosis-surviving Fas-deficient cells compared to B6 T cells. To overcome these differences in cell death between control and B6/*lpr* T cells, we used the cell death inhibitor zVAD, a broad-spectrum caspase inhibitor that blocks caspase-8 processing at the DISC and thus inhibits transduction of apoptotic signaling (Scheme 2, Introduction). Cell cycle analysis showed that in the presence of this inhibitor, B6/*lpr* T cells still hyperproliferated compared to controls (Fig. 3A, bottom). This was also demonstrated by increased [^{3}H]thymidine uptake (Fig. 3B) and accumulation of live cells in the culture (Fig. 3C).

To further confirm that B6/*lpr* T cell hyperproliferation after secondary stimulation was due to lack of proliferation control by Fas rather than to the increased number of live T cells in B6/*lpr* *vs.* B6 cultures, we examined division rates of CFSE-labeled B6/*lpr* *vs.* B6 T cells. In this assay, incorporated fluorescent marker is reduced by half the initial intensity after each cell division and can be monitored by flow cytometry. CFSE dilution of propidium iodide-negative (PI^-) live B6/*lpr* cells was greater compared to B6 counterparts (Fig. 3D, top). zVAD treatment (50 μM) had no effect on the proliferative/division potential of B6 T cells (Fig. 3D, bottom). The CFSE dilution data showed total population fluorescence shifts, rather than typical sequential dilution peaks. This is probably an inherent characteristic of CFSE labeling of repeatedly stimulated cells, as CFSE staining of naïve T cells after the first stimulation showed sequential dilution peaks (Fig. 2B). We also analyzed expression of the Ki-67 proliferation marker in CD4+ T cells (Katzman et al., 2010), and found a notable increase in expression of this marker 24 h after secondary stimulation (Fig. 3E). Restimulated Fas-deficient T cells thus divided at an increased rate compared to apoptosis-surviving B6 T cells, supporting the premise that Fas has a proliferation-regulating effect on T cells after repeated stimulation.

Hyperproliferation of B6/*lpr* T cells might reflect an increased cell activation

status; we therefore examined the impact of Fas deficiency on T cell activation. In accordance with the observed enhanced proliferation of B6/*lpr* T cells, we detected more IL-2 production (Fig. 3F), used as a surrogate for active proliferative signaling (Bécart et al., 2007; Alshamsan et al., 2011). Finally, we found that at 24 h after second stimulation in the presence of zVAD, these cells showed greater expression of CD25 (Fig. 3G, top) and CD69 (Fig. 3G, bottom).

These results thus indicate that after secondary stimulation, B6/*lpr* T cells reach higher activation status than B6 T cells, and this justify their hyperproliferative phenotype.

Fas controls rechallenged T cell proliferation independently of its pro-apoptotic function

To confirm that Fas control of proliferation is independent of AICD-inducing potential and to exclude possible secondary effects of zVAD, we analyzed proliferation of restimulated B6 and B6/*lpr* T cells in apoptosis-free conditions. We established in our laboratory two apoptosis-free protocols for the analysis of T cell proliferation after repeated stimulation (Scheme 6, Materials and Methods).

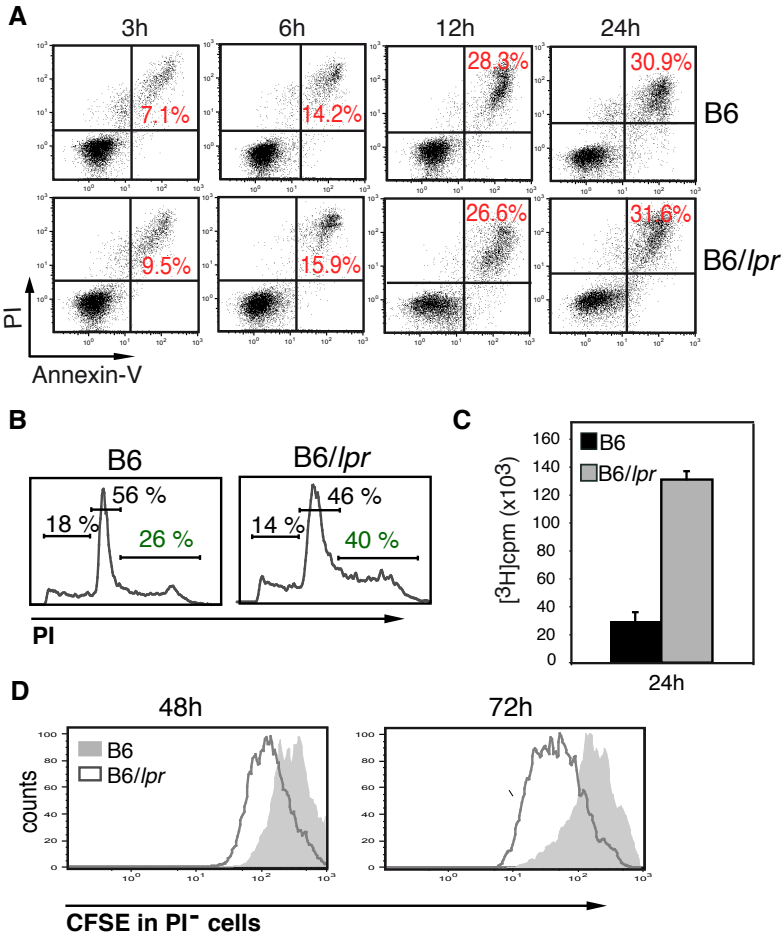


Figure 4. Fas controls T cell proliferation independently of its AICD potential. A. Live cells were gradient-separated 48 h after secondary challenge, ConA-restimulated and annexin-V and PI double staining was performed at indicated times; similar cell death ratios were observed for B6 and B6/*lpr* T cells. Representative data are shown (n = 2). **B.** Cell cycle analysis at 24 h after the third stimulation showed elevated proliferation of B6/*lpr* T cells compared to controls in similar conditions of cell death. Representative histograms are shown (n = 2). **C.** Hyperproliferation of B6/*lpr* T cells in apoptosis-free conditions, measured by [^{3}H] thymidine uptake at 24 h after the third challenge. Values show mean ± SD (n = 4). **D.** Dilution rate of CFSE-labeled PI-negative control and B6/*lpr* T cells at 48 h and 72 h after the third stimulation. Representative data are shown (n = 2).

In the first system, at 48 h after secondary stimulation, B6 and B6/*lpr* T cells were gradient-purified to separate live from apoptotic cells (>90% efficiency) and live cells were immediately restimulated with ConA. This restimulation did not induce apoptosis in B6 or B6/*lpr* T cells, as determined by Annexin V and PI staining (Fig. 4A), but led to marked B6/*lpr* T cell hyperproliferation 24 h after the third ConA stimulation (Fig. 4B). Enhanced proliferation was also evident in [^{3}H]thymidine incorporation assays (Fig. 4C) and CFSE dilution experiments (Fig. 4D). These data confirm that B6/*lpr* T cell hyperproliferation is not a direct result of an apoptosis defect, but a consequence of each cell's greater capacity to divide.

In the second protocol, restimulated and gradient-purified cells were cultured with IL-2. Cell cycle analysis showed that cell death was also minimal in this case and was the same for both cell groups (Fig. 5A). In contrast, B6/*lpr* T cells hyperproliferated compared to controls, as shown by [^{3}H]thymidine incorporation (Fig. 5B) and CFSE dilution (Fig. 5C). These observations indicate that hyperproliferative characteristics of B6/*lpr* T cells are maintained, even for prolonged periods post-restimulation, and show that Fas-deficient cells have greater division capacity, which permits them to extend the proliferative response beyond the immediate reaction to TCR stimulation. Overall, data from apoptosis-free systems demonstrate that Fas regulates T cell proliferation independently of its AICD-inducing potential.

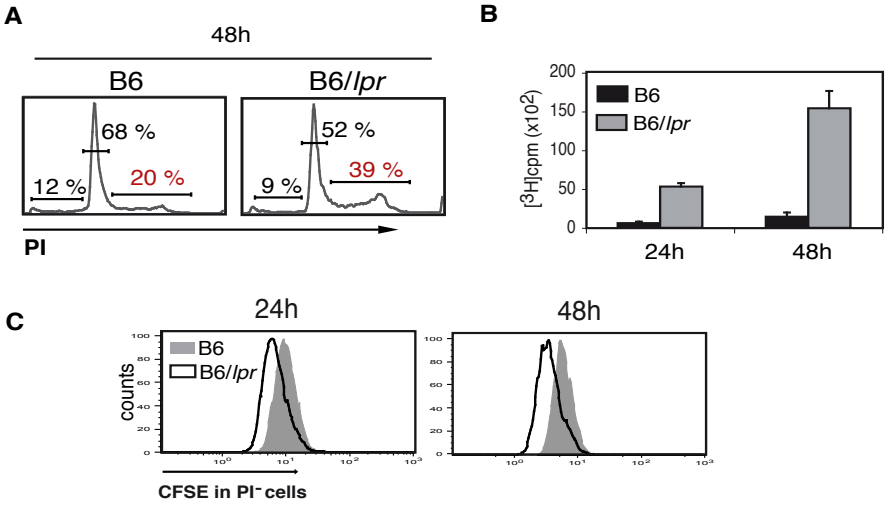


Figure 5. B6/*lpr* T cell hyperproliferation in an apoptosis-free proliferation system
A. Live cells were gradient-separated at 48 h after secondary stimulation and expanded with IL-2 for another 48 h. PI staining and cell cycle analysis showed enhanced proliferation of B6/*lpr* T cells compared to controls. **B.** Proliferation of live B6 and B6/*lpr* T cells in an apoptosis-free system was measured by [^{3}H]thymidine incorporation at 24 h and 48 h after gradient separation and the third stimulation (mean ± SD, n = 4). **C.** CFSE dilution experiments confirmed elevated B6/*lpr* T cell proliferation compared to controls in minimal cell death conditions. In all cases, representative data are shown (n = 2).

Fas-dependent control of T cell proliferation requires interaction with Fas ligand

As Fas interaction with its ligand is necessary to induce the apoptosis signaling pathway, we analyzed whether this interaction is needed for Fas-dependent control of cellular proliferation. We used B6/*gld* T cells that express fully functional Fas, but lack Fas ligand and develop autoimmune disorders similar to those of B6/*lpr* mice (Lynch et al., 1994; Ramsdell et al., 1994). We subjected CD4+ T cells from B6/*gld* mice to the repeated stimulation protocol and observed by means of [^{3}H]thymidine incorporation and CFSE dilution experiments that, like B6/*lpr* T cells, they hyperproliferated compared to controls (Fig. 6A). These results indicated that Fas-FasL interaction is important in proliferation control. Similar to B6/*lpr* T cells, B6/*gld* cells did not undergo apop-

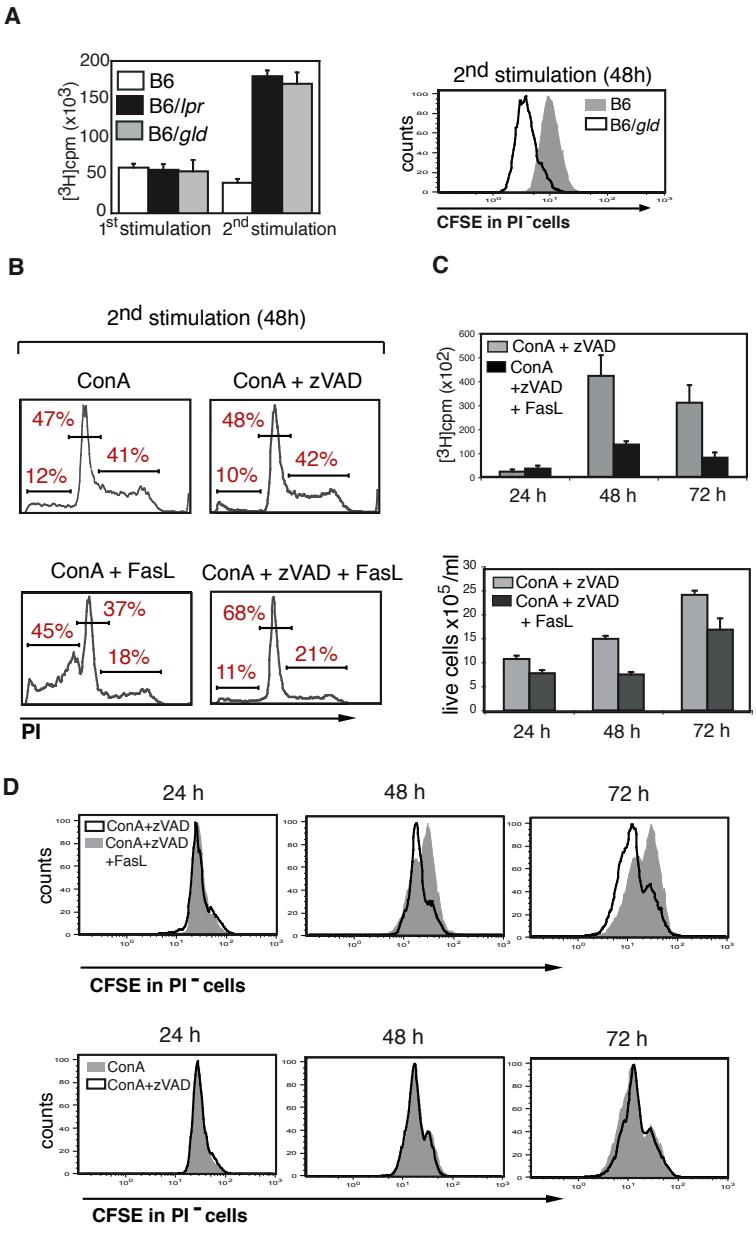


Figure 6. Fas interaction with its ligand directly attenuates T cell proliferation

A. FasL-deficient B6/*gld* T cells, like B6/*lpr* T cells, hyperproliferate after secondary stimulation, as detected by [^{3}H]thymidine incorporation and CFSE dilution assys. **B.** Cell cycle and apoptosis analysis of ConA-restimulated B6/*gld* T cells treated with recombinant (r)FasL (150 ng/ml) or rFasL + zVAD (150 ng/ml and 50 μM, respectively). Representative histograms are shown (n = 4). **C.** FasL treatment of restimulated B6/*gld* T cells decreased their hyperproliferation ([^{3}H]thymidine incorporation, top) and as a result, the number of live cells in culture (trypan blue staining, bottom). Values show mean ± SD (n = 4). **D.** Reduced proliferation of FasL-treated B6/*gld* T cells after second stimulation, as determined by CFSE dilution (top). Addition of zVAD (50 μM) had no effect on restimulated B6/*gld* T cell proliferation (bottom). Representative data are shown (n = 4).

tosis, as determined by PI staining (Fig. 6B, top).

To test whether restored Fas-FasL interaction can reduce proliferation, we treated Fas-expressing B6/*gld* T cells with 150 ng/ml of recombinant FasL (rFasL) and tested whether hyperproliferation was reduced after the second challenge. As rFasL addition induced apoptosis of ConA-restimulated B6/*gld* T cells (Fig. 6B, bottom left), we used the inhibitor zVAD (50 μM) to avoid cell death and to allow observation of the Fas effect on cell proliferation alone. zVAD treatment efficiently inhibited rFasL-induced apoptosis (Fig. 6B, bottom right), and rFasL addition notably decreased B6/*gld* cell hyperproliferation after secondary stimulation (Fig. 6B, bottom right). This result was confirmed in a [^{3}H]thymidine incorporation assay and by counting live cell numbers (Fig. 6C). CFSE dilution experiments also showed a decreased division rate for B6/*gld* T cells treated with rFasL, indicating that Fas-FasL interaction has a direct effect on the proliferation of restimulated T cells (Fig. 6D, top); a control showed that 50 μM zVAD had no effect on T cell proliferation (Fig. 6D, bottom).

Overall, these data demonstrate that, independently of its AICD-inducing effect, Fas interaction with FasL directly attenuates restimulated B6/*gld* T cell hyperproliferation.

Hyperproliferation of Fas-deficient cells is not due to higher number of memory T cells entering the second stimulation

To rule out the possibility that B6/*lpr* T cell hyperproliferation characterizes distinct initial activation/memory status between B6 and B6/*lpr* cells or that culture conditions favor selection of Fas-deficient cell clones with the initial high proliferative capacity, we sorted spleen naïve (CD44low/CD62Lhigh) and memory (CD44high/CD62Llow) CD4+ T cells from B6 and B6/*lpr* mice (Fig. 7A). Cells were subjected to the standard AICD protocol. Following the first stimulation, proliferation of B6/*lpr* CD44low/CD62Lhigh T cells (the term “naïve” can not be use at this stage, as “naïve” refers to cells that have never seen antigen) was in fact lower than that of control T cells, as determined by cell cycle analysis (Fig. 7B). This finding concurs with reports that Fas provides necessary costimulation for naïve T cells (Alderson et al., 1993; Maksimow et al., 2006; Rethi et al., 2008). In addition, there were no differences between B6 and B6/*lpr* T cells in expression of activation markers CD25 and CD69 (Fig. 7C).

Following the second stimulation, however, CD44low/CD62Lhigh B6/*lpr* T cells showed much higher CD25 expression than control T cells. This was also the case of CD44high/CD62Llow B6/*lpr* T cells, although here the difference

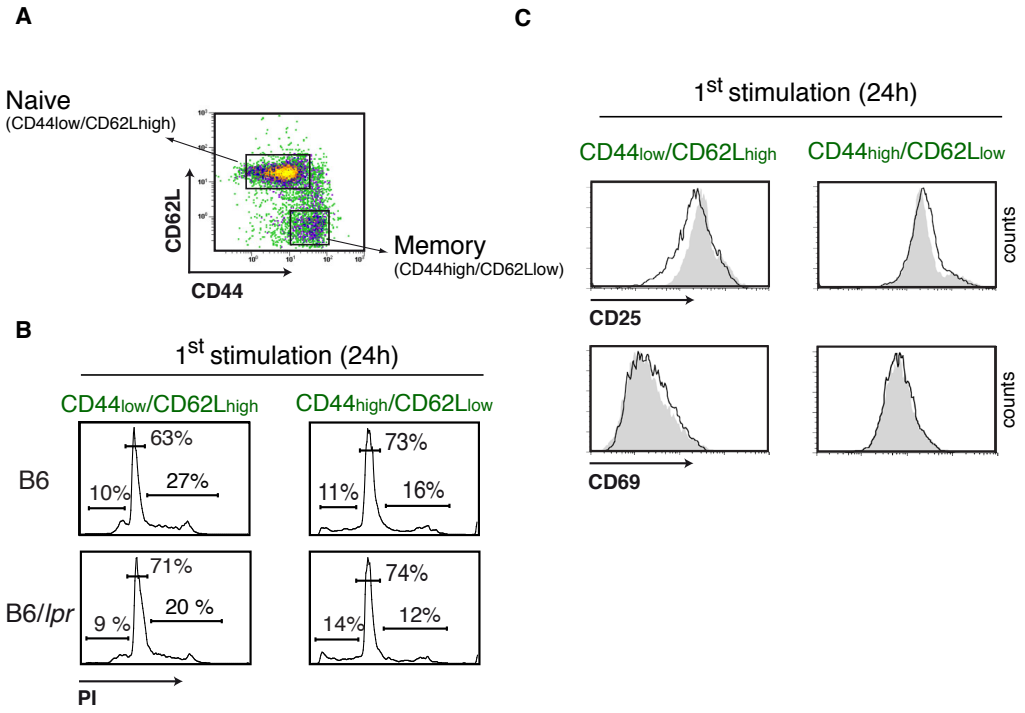


Figure 7. Proliferative response of sorted naïve and memory T cells after first stimulation. **A.** Spleen-derived cells from B6 and B6/*lpr* T mice were stained for CD4, CD44, CD62L surface markers, and naïve (CD44lowCD62Lhigh) and memory T cells (CD44highCD62Llow) were sorted. **B.** Cell cycle analysis at 24 h after primary stimulation showed similar proliferation ratios between B6 and B6/*lpr* CD44low/CD62Lhigh cells and between B6 and B6/*lpr* CD44high/CD62Llow T cells. **C.** Surface expression of CD25 and CD69 activation markers on sorted B6 and B6/*lpr* T cell populations. In all cases, representative data are shown (n=3).

was less notable (Fig. 8A, top). Surface CD69 expression was also considerably higher in CD44low/CD62Lhigh B6/*lpr* T cells, which was not the case for CD44high/CD62Llow B6/*lpr* T cells (Fig. 8A, bottom).

We analyzed proliferation of both cell subsets by testing expression of the proliferation marker Ki-67 and by cell cycle analysis. Ki-67 was upregulated in CD44low/CD62Lhigh B6/*lpr* T cells (Fig. 8B). In agreement with this result, cell cycle analysis showed markedly higher proliferation of B6/*lpr* CD44low/CD62Lhigh T cells compared to this subset in B6 mice (Fig. 8C, left), indicating that these cells have a greater intrinsic proliferative capacity. The proliferation rate of CD44high/CD62Llow was also increased in B6/*lpr* compared to B6 mice (Fig. 8C, right); in both B6/*lpr* and B6 T mice, CD44high/CD62Llow proliferation was lower than that of CD44low/CD62Lhigh T cells.

Overall, these results exclude the possibility that B6/*lpr* T cell hyperproliferation is due to differences in activation/memory status before second stimulation, or to artifactual culture conditions that favor selection of Fas-deficient cell clones

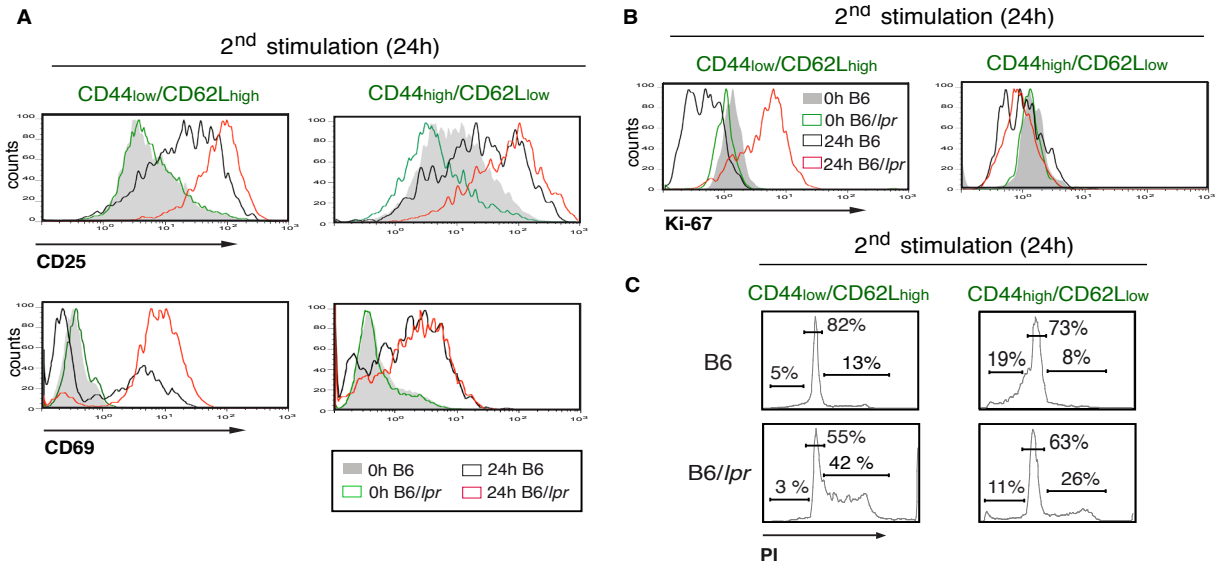


Figure 8. Hyperactivation and hyperproliferation of sorted naïve and memory B6/*lpr* T cells after secondary stimulation. **A.** Increased surface expression of CD25 on CD44low/CD62Lhigh and CD44high/CD62Llow B6/*lpr* T cells (top) and increased CD69 expression on CD44low/CD62Lhigh B6/*lpr* T cells (bottom). **B.** Enhanced proliferation of CD44low/CD62Lhigh B6/*lpr* T cells compared to CD44low/CD62Lhigh B6 T cells, determined by Ki-67 expression. **C.** Cell cycle analysis of sorted populations showed hyperproliferation of CD44low/CD62Lhigh and CD44high/CD62Llow B6/*lpr* T cells, as well as notable differences in the proliferation ratio between sorted populations from each mice genotype. In all cases, representative data are shown (n = 3).

with initial high proliferative capacity. B6/*lpr* T cells hyperproliferate following re-stimulation even when they show naïve T cell status at the beginning of the culture period, which confirms the role of Fas in direct control of T cell proliferation after repeated antigenic stimulation.

p21 overexpression in B6/*lpr* mice reduces *in vivo* T cell hyperproliferation and lymphadenopathy development

Our *in vitro* data thus far indicated that Fas negatively controls T cell proliferation. As the etiology of the B6/*lpr* mouse phenotype has not been explained to date, we considered that the lack of Fas control of proliferation might modulate the B6/*lpr* phenotype. We therefore explored the physiological relevance of T cell hyperproliferation in autoimmunity development in Fas-deficient mice. We used an

in vivo model generated in our laboratory in which the cell cycle inhibitor p21 is overexpressed in the B6/*lpr* mouse T cell compartment.

We previously described p21 as a suppressor of autoimmunity (Balomenos et al., 2000; Arias et al., 2007), and showed that it does not affect primary T cell proliferation, but controls proliferation of apoptosis-surviving T cells after secondary antigen challenge (Arias et al., 2007). This led us to speculate that p21 overexpression in B6/*lpr* mice might reduce the autoimmune disorder in these animals, since their T cells hyperproliferate after restimulation.

B6/*lpr*-p21tg mice were generated from C57BL/6-p21tg mice (B6-p21tg); p21 transgene expression in these animals is restricted to T cells by the proximal Lck promoter. B6-p21tg mice show normal T cell development and differentiation (Fotedar et al., 1999).

In vivo T cell proliferation is similar to that of B6 mice (Fig. 9A), as determined by BrdU incorporation by lymph node CD4+, CD8+ and DN T cells. We found that although the p21 transgene had no effect on B6 T cell proliferative characteristics, it greatly inhibited (>2-fold) CD4+, CD8+ and DN T cell hyperproliferation in B6/*lpr*-p21tg mice (Fig. 9B).

These results indicated an antiproliferative effect of p21 overexpression on peripheral B6/*lpr*-p21tg T cells. To rule out a possible p21 transgene effect on selection events and development in the thymus, we examined the T cell profiles in B6, B6/*lpr*, B6-p21tg and B6/*lpr*-p21tg mouse thymus. The similarity of frequencies for all thymocyte subsets in all genotype groups supports the view that p21 transgene function is manifested in peripheral B6/*lpr*-p21tg T cells (Fig. 10).

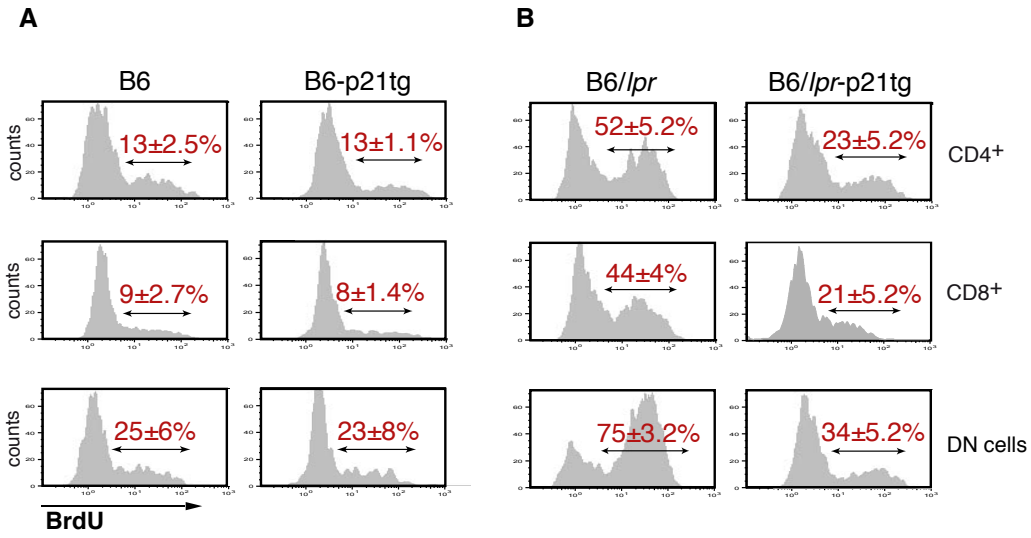


Figure 9. p21 overexpression inhibits *in vivo* hyperproliferation of B6/*lpr* T cells
A. *In vivo* BrdU incorporation by lymph node T cells from 2-month-old B6, B6-p21tg and **B.** B6/*lpr*, B6/*lpr*-p21tg mice shows decreased proliferation for B6/*lpr*-p21tg CD4+, CD8+ and DN T cells. Values are mean ± SD (n = 4).

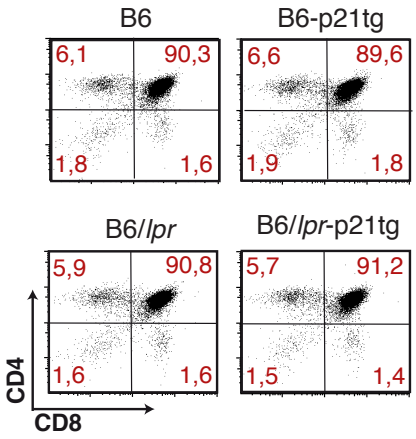


Figure 10. Thymic T cell profile for B6, B6/*lpr*, B6-p21tg and B6/*lpr*-p21tg mice
Single-cell suspensions from the thymus of 1.5-month-old B6, B6/*lpr*, B6-p21tg and B6/*lpr*-p21tg mice were prepared, and surface expression of CD4 and CD8 analyzed by flow cytometry. Representative histograms are shown (n = 3).

Reduced lymphadenopathy and autoimmune manifestations in B6/*lpr*-p21tg mice

B6/*lpr* mice develop lymphadenopathy and splenomegaly, which originate from accumulation of DN (Fig. 11A, left) and CD4+ memory T cells (Fig. 12A). Expansion of these cell subsets is evident at two months of age and increases with time. We found that p21 overexpression led to a clear reduction of the DN T cell population in B6/*lpr*-p21tg mice (Fig. 11A, top), probably as a result of the reduced *in vivo* proliferation of DN T cells in B6/*lpr*-p21tg mice (Fig. 9B, bottom). The DN T cell population in B6-p21tg mice was unaffected by overexpression of the transgene (Fig. 11A, bottom). In accordance with these results, we observed a notable reduction in lymphadenopathy in B6/*lpr*-p21tg mice (Fig. 11B). We detected no endogenous p21 expression in DN T cells; this

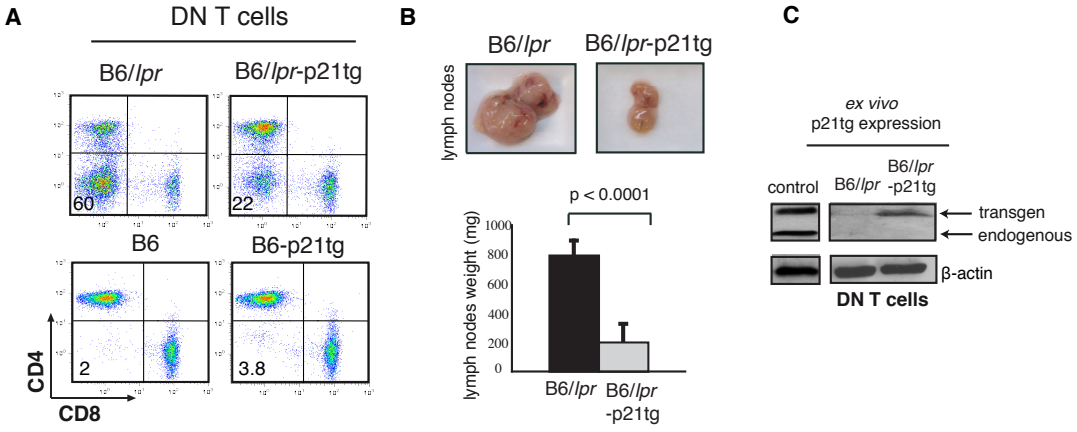


Figure 11. Decreased DN T cell accumulation and reduced lymphadenopathy in B6/*lpr*-p21tg mice. **A.** Decreased proportions of DN T cells in lymph nodes of 5-month-old B6/*lpr*-p21tg compared to B6/*lpr* mice. Values are mean ± SD (n = 8). **B.** Decreased size (top) and weight (bottom) of cervical lymph nodes in 8 month-old female B6/*lpr*-p21tg *vs.* B6/*lpr* mice. Values are mean ± SD (n = 10, $p<1 \times 10^{6}$). **C.** Western blot reflecting expression of transgenic p21 in DN T cells from 2-month-old mice. Endogenous p21 is not expressed in DN T cells compared to control CD4+ T cells cultured *in vitro* in the presence of IL-2. β-actin was used as a loading control.

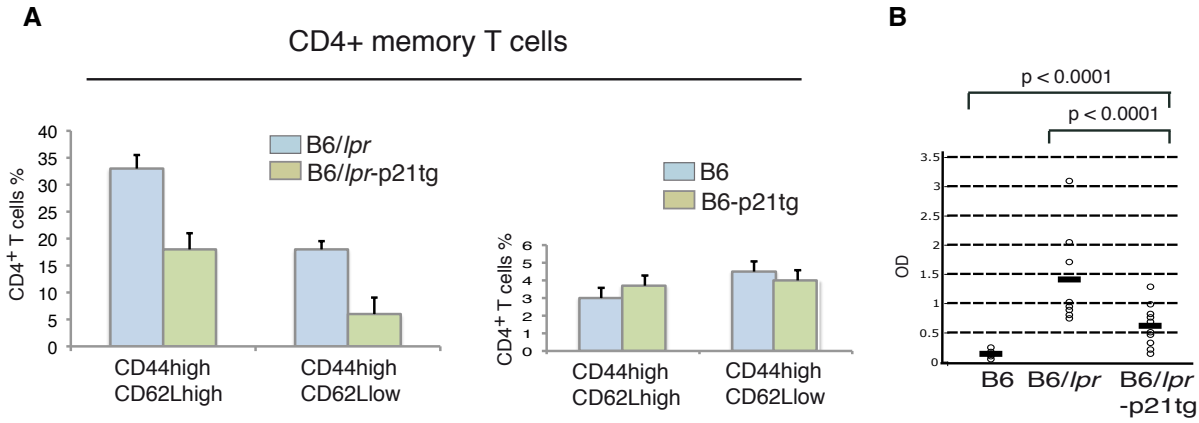


Figure 12. p21 overexpression reduces manifestations of autoimmune disease
A. Proportions of lymph node memory T cells (CD44high, CD62Lhigh/low) from 2 month-old B6/*lpr*, B6/*lpr*-p21tg, B6 and B6-p21tg mice. p21 overexpression greatly reduces age-related memory T cell accumulation in B6/*lpr* mice. Values are mean ± SD (n = 5 mice/group or genotype). **B.** Relative levels of anti-DNA IgG detected by ELISA in 4-month-old B6, B6/*lpr* and B6/*lpr*-p21tg mice. Values are mean ± SD (n = 9 mice/group or genotype, p=1 x 10^5).

lack of p21 probably explains the hyperproliferation of these cells, whereas expression of the p21 transgene reduces this elevated proliferation (Fig. 11C).

In addition to decreasing lymphadenopathy, p21 overexpression also ameliorated manifestations of the autoimmune disease; it considerably reduced the expansion of both subsets (CD44high/CD62Lhigh or low) of effector/memory T cells in B6/*lpr*-p21tg mice (Fig. 12A) and led to a notable drop in serum anti-DNA antibody levels in B6/*lpr*-p21tg mice compared to B6/*lpr* controls (Fig. 12B).

T cell-directed expression of the p21 transgene therefore has a dual effect in B6/*lpr* mice; on the one hand, it reduces DN T cell expansion and lymphadenopathy, and on the other, it ameliorates autoimmune manifestations.

p21 overexpression diminishes lupus-like autoimmunity and death incidence in MRL/*lpr* mice

Our results demonstrated that p21 overexpression in B6/*lpr*-p21tg T cells effectively limits the autoimmunity symptoms and lymphadenopathy of B6/*lpr* mice. We next tested whether p21 hyperexpression had similar potential to inhibit the severe, death-inducing autoimmunity features in the MRL/*lpr* mice; the phenotype of these animals is a result of the combined autoimmunity-prone MRL background and the *lpr* mutation.

We found that T cell-directed p21 hyperexpression reduced the proportion

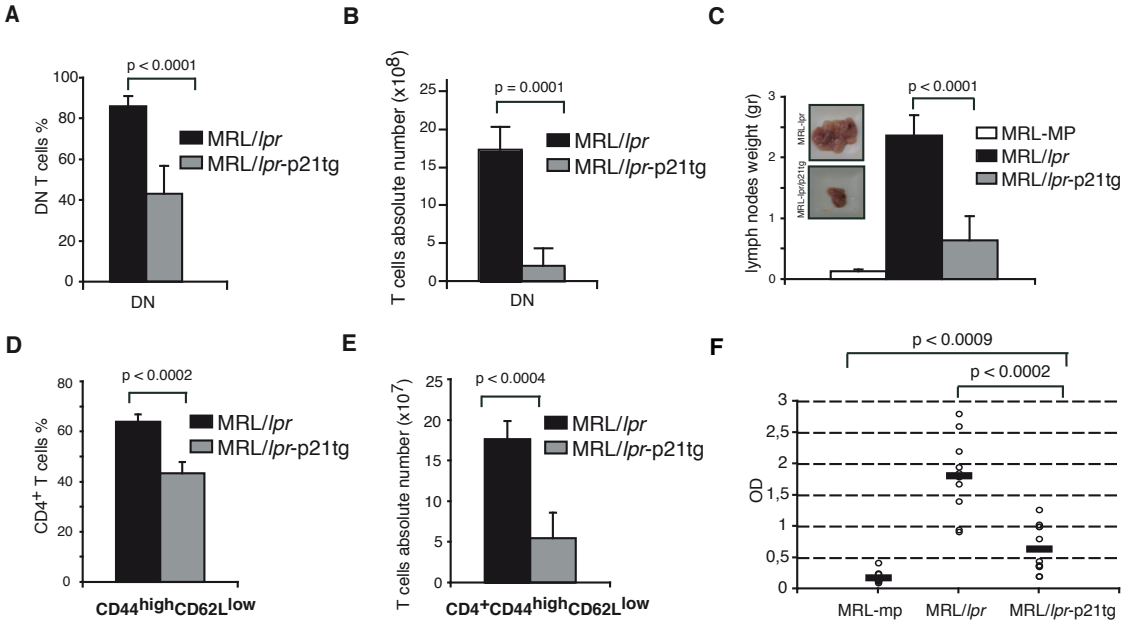


Figure 13. Decreased DN and memory T cell accumulation, lymphadenopathy and anti-DNA IgG production in MRL/*lpr*-p21tg mice. **A.** Percentage of lymph node DN T cells normalized to CD4+ T cells in 4- to 6-month-old MRL/*lpr* and MRL/*lpr*-p21tg mice. Values are mean ± SD (n = 10, p=4,24 x 10^5). **B.** Absolute number of DN T cells in lymph nodes of 4- to 6-month-old MRL/*lpr* and MRL/*lpr*-p21tg mice. Values are mean ± SD (n = 10, p=1 x 10^4). **C.** Decreased size and weight of cervical lymph nodes in 4- to 6 month-old female MRL/*lpr*-p21tg *vs* MRL/*lpr* and MRL-MP mice. Values are mean ± SD (n = 10, p=1.7 x 10^6). **D.** Percentage of memory T cells (CD44high, CD62Llow) in 4- to 6 month-old MRL/*lpr*-p21tg and MRL/*lpr* mice normalized to CD4+ T cells. Values are mean ± SD (n = 8, p=0.24 x 10^4). **E.** Absolute number of memory (CD44high, CD62Llow) T cells in lymph nodes of 4- to 6-month-old MRL/*lpr* and MRL/*lpr*-p21tg mice. Values are mean ± SD (n = 8, p=0.43 x 10^4). **F.** Relative levels of anti-DNA IgG detected by ELISA in 4-month-old MRL-MP, MRL/*lpr* and MRL/*lpr*-p21tg mice. Values are mean ± SD (n = 9 mice/group or genotype, p<9 x 10^5).

(Fig. 13A) and absolute number (Fig. 13B) of lymph node DN T cells, as well as lymphadenopathy in MRL/*lpr*-p21tg compared to MRL/*lpr* mice (Fig. 13C). The weight of lymph nodes from transgenic mice was markedly decreased, and dropped to levels similar to those of MRL-MP mice, which are not prone to autoimmunity.

Compared to controls, MRL/*lpr*-p21tg mice also showed a reduction in the proportion (Fig. 13D) and absolute number (Fig. 13E) of effector/memory T cells in lymph nodes, and a strong decrease in serum anti-DNA antibody levels, which decreased almost to the level of the MRL-MP controls (Fig. 13F). As MRL/*lpr* mice have much higher levels of anti-DNA antibodies than B6/*lpr* mice, it appears that p21 overexpression is able to control the predisposition of MRL/*lpr* mice to severe autoimmunity. Indeed, when compared to MRL/*lpr* mice, MRL/*lpr*-p21tg mice showed clear improvement in critical aspects of lupus-like kidney disease, such as lower immune complex deposition,

diminished inflammatory infiltration of CD4+ T cells and F4/80+ macrophages in kidney (Fig. 14A), and reduced glomerulonephritis development (Fig. 14B). The ameliorating effect of the p21 transgene on disease manifestations in MRL/*lpr* mice is translated into survival of MRL/*lpr*-p21tg mice (Fig. 14C).

Overall, these data show that p21 is a potent suppressor of autoimmunity, as it can restrain the autoimmune symptoms and death in the MRL/*lpr* mouse models that develop accelerated, severe lupus-like disease.

p21 overexpression does not restore apoptosis defect in death-deficient B6/*lpr* T cells

To further analyze the role of p21 as a proliferation-attenuator of B6/*lpr*-p21tg T cells, we performed *in vitro* stimulation and proliferation studies. Previous work from our laboratory with p21-deficient mice attributed a role to p21 in controlling the expansion of effector/memory T cells, but not of naïve T cells (Arias et al., 2007).

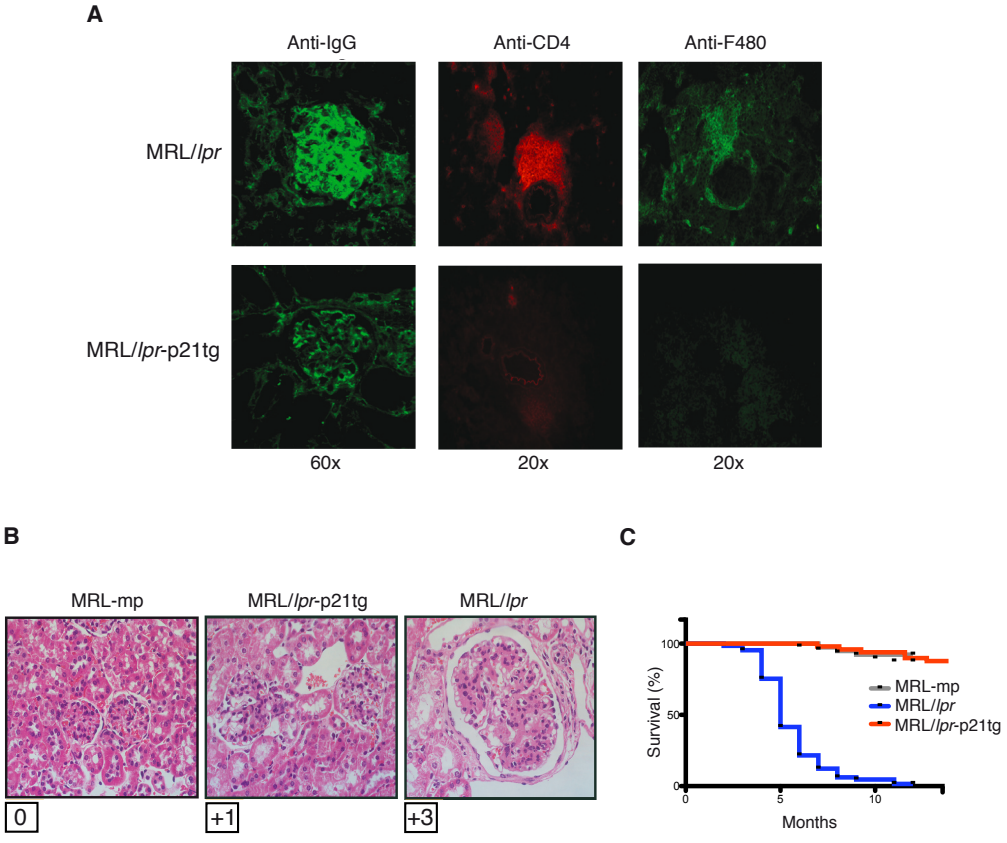


Figure 14. Diminished lupus-like autoimmunity and death in MRL/*lpr*-p21tg mice
A. Anti-IgG, -CD4 and -F/480 immunostaining of kidney cryosections from 6-month-old MRL/*lpr* and MRL/*lpr*-p21tg mice. **B.** Analysis of glomeruli in representative kidney sections from 6-month-old MRL-MP, MRL/*lpr* and MRL/*lpr*-p21tg mice. Glomerulonephritis grade (based on the Berdem scale) is shown. **C.** Kaplan-Meier survival curves indicating the impact of the p21 transgene on MRL/*lpr*-p21tg mouse lifespan.

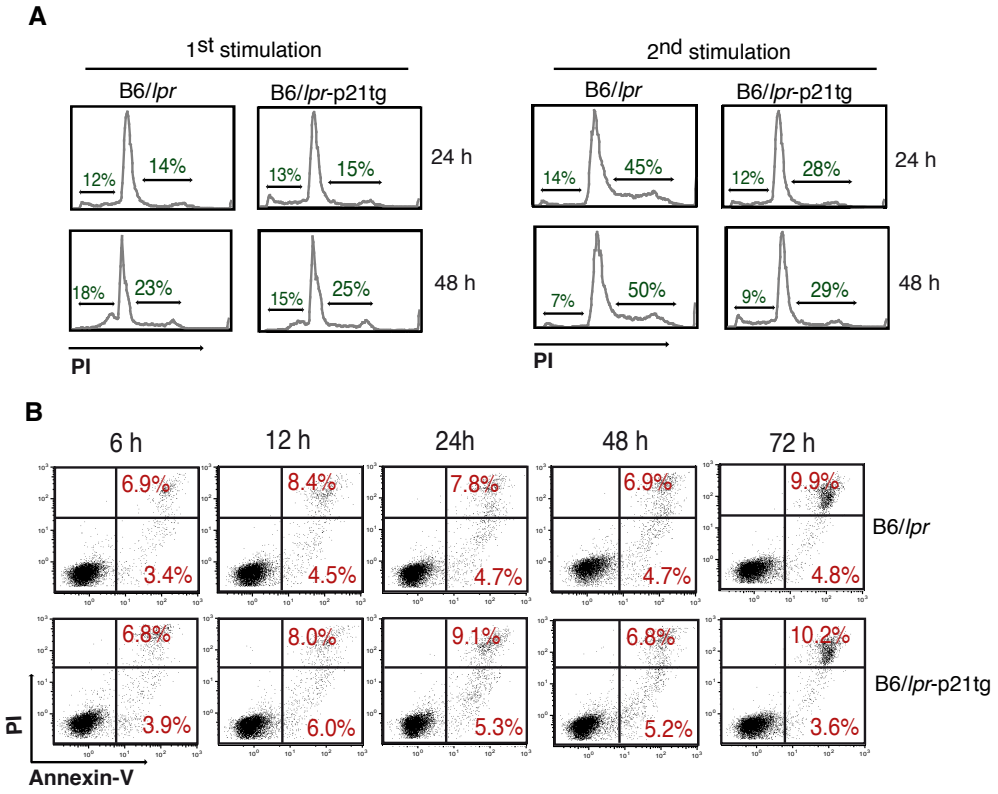


Figure 15. Apoptosis defect in B6/*lpr* T cells is unaffected by p21 overexpression
A. Cell cycle analysis at 24 h after the first (left) and the second (right) stimulation showed decreased proliferation of B6/*lpr*-p21tg T cells after secondary stimulation compared to controls in similar conditions of cell death. Representative histograms are shown (n = 3).
B. Annexin-V and PI double staining at various times after second stimulation showed the same cell death defect in B6/*lpr* and B6/*lpr*-p21tg T cells. Representative histograms are shown (n = 2).

Here we examined whether p21 overexpression affected B6/*lpr*-p21tg T cell proliferation compared to B6/*lpr* controls after primary and secondary stimulation. Spleen-derived T cells from B6/*lpr*-p21tg and B6/*lpr* mice were ConA-stimulated, expanded briefly in IL-2 (2 days), and then restimulated. PI staining and cell cycle analysis showed that p21 overexpression had no effect on T cell proliferation following primary stimulation (Fig. 15A, left), whereas it clearly reduced cell cycle progression of B6/*lpr*-p21tg T cells at 24 and 48 h after secondary stimulation compared to B6/*lpr* T cells (Fig. 15A, right).

Following primary ConA stimulation, IL-2-dependent culture, and secondary activation of T cells, the Fas/FasL system is activated and apoptosis proceeds. Fas-deficient B6/*lpr* T cells have a defect in apoptosis after secondary T cell stimulation. The observed reduction in B6/*lpr*-p21tg T cell proliferation *in vivo*, and *in vitro* post-restimulation, might be caused by a proliferation inhibitory effect of p21. Alternatively, this reduction could be due to p21 transgene-induced

restoration of defective apoptosis in B6/*lpr* T cells. Nonetheless, the absence of an apoptotic hypodiploid peak in cell cycle profiles after secondary stimulation of both B6/*lpr*-p21tg and B6/*lpr* T cells indicated that p21 overexpression does not restore the B6/*lpr* apoptotic defect (Fig. 15A, right). To further rule out a role for p21 as an apoptosis inducer in B6/*lpr* T cells after secondary stimulation, we analyzed apoptosis induction in B6/*lpr* and B6/*lpr*-p21tg T cells by annexin-V and PI double staining. The annexin-V(+) PI(-) population represents apoptotic T cells and the annexin-V(+)PI(+) subset indicates post-apoptotic, dead T cells. We found that p21 overexpression did not interfere with the defective apoptosis of B6/*lpr* T cells, as annexin-V staining showed similarly deficient apoptosis for B6/*lpr* and B6/*lpr*-p21tg T cells, at both early and late times post-secondary simulation (Fig. 15B).

These results therefore exclude a possible apoptosis-inducing effect of the p21 transgene in the reduction of T cell proliferation observed in B6/*lpr*-p21tg mice. The data therefore suggest that p21 overexpression has an anti-proliferative effect on B6/*lpr*-p21tg T cells, and limits B6/*lpr*-dependent T cell hyperproliferation without altering the apoptosis defect.

Reduced B6/*lpr*-p21tg T cell proliferation after secondary *in vitro* stimulation

The observed reduction in B6/*lpr*-p21tg T cell proliferation only after secondary stimulation (Fig. 15A) was further confirmed by [^{3}H]thymidine uptake at 48 h after the first and second ConA stimulation. Following primary challenge, we found similar proliferation rates for B6/*lpr* and B6/*lpr*-p21tg T cells (Fig. 16A). In contrast, after secondary stimulation, we observed a sharp decrease (>50%) in [^{3}H]thymidine uptake by B6/*lpr*-p21tg compared to B6/*lpr* T cells (Fig. 16A). The capacity of p21 overexpression to reduce cell cycle progression of restimulated B6/*lpr*-p21tg T cells was further validated by the decreased expression of the Ki-67 proliferation marker by B6/*lpr*-p21tg *vs.* B6/*lpr* T cells (>3-fold) after secondary stimulation (Fig. 16B).

To further confirm that p21 overexpression reduced the capacity of T cells to divide, we performed CFSE dilution experiments. Fluorescence intensity dilution of CFSE-labeled B6/*lpr*-p21tg and B6/*lpr* T cells established that p21 overexpression did not affect primary proliferation of T cells (Fig. 16C, left). In accordance with previous data, we nonetheless observed decreased fluorescence intensity dilution of CFSE-labeled B6/*lpr*-p21tg T cells compared to B6/*lpr* T cells, which was detectable at 48 h and became clear 72

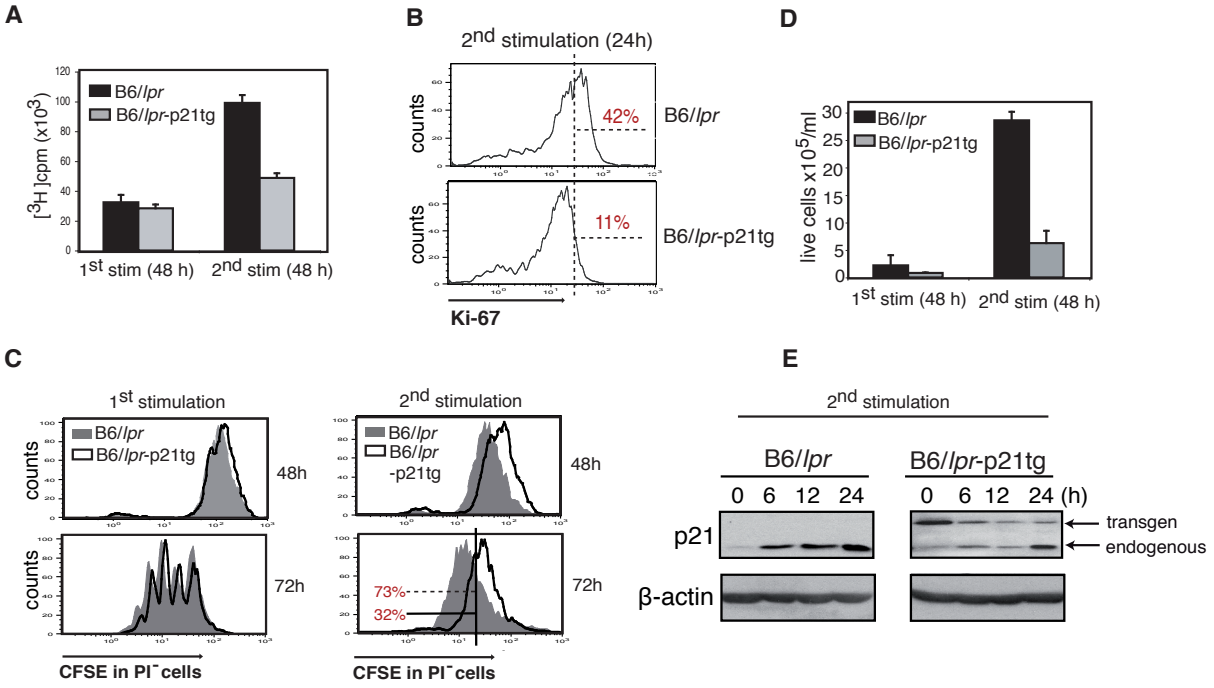


Figure 16. p21 overexpression decreases *in vitro* B6/*lpr* cell hyperproliferation after secondary stimulation. **A.** Purified CD4+ T cells from B6/*lpr* and B6/*lpr*-p21tg mice were ConA-stimulated (1st stimulation) or restimulated with ConA after an IL-2 expansion phase (2nd stimulation). The p21 transgene reduced T cell responses only after secondary stimulation. Proliferation was measured by [^{3}H]thymidine uptake at 48 h after first or second challenge. Values show mean ± SD (n = 4). **B.** B6/*lpr*-p21tg T cells showed reduced expression of the Ki-67 proliferation marker compared to B6/*lpr* T cells at 24 h after secondary stimulation, as assessed by flow cytometry analysis. Representative histograms are shown (n = 2). **C.** CFSE dilution rate of gated PI-negative T cells, following the first (left) and the second (right) ConA stimulation. Representative histograms are shown (n = 3). **D.** Decreased generation of B6/*lpr*-p21tg CD4+ T cells compared to B6/*lpr* T cells after secondary stimulation, as determined by Trypan blue dye exclusion. Values show mean ± SD (n = 4). **E.** Western blot reflecting the expression of endogenous and transgenic p21 in CD4+ T cells from 2-month-old B6/*lpr* and B6/*lpr*-p21tg mice. Spleen cells were isolated, ConA stimulated, IL-2 expanded and restimulated for indicated times. β-actin was used as a loading control.

h after secondary stimulation (Fig. 16C, right). Restimulated B6/*lpr*-p21tg T cells thus divided at a decreased rate compared to B6/*lpr* T cells. The CFSE dilution results were supported by the severe reduction (>5-fold) in the absolute number of live T cells in B6/*lpr*-p21tg *vs*. B6/*lpr* T cell cultures at 48 h post-secondary activation (Fig. 16D). As anticipated, we detected comparable numbers of these two cell types at the termination of primary stimulation (Fig. 16D). These data indicate that the p21 transgene has a regulatory role in the expansion of B6/*lpr*-p21tg T cells after *in vitro* secondary, but not after primary challenge.

To further evaluate the effect of p21 overexpression on T cell proliferation after secondary stimulation, we examined expression of the p21 transgene and of

endogenous p21 by Western blot. We found that, at the end of the IL-2 expansion step (0 h, Fig. 16E), endogenous p21 was minimally expressed in B6/*lpr* and B6/*lpr*-p21tg T cells, whereas p21 transgene expression was high in the latter. After the second activation step, endogenous p21 was greatly induced in B6/*lpr* T cells, suggesting that p21 is critical for the control of proliferation in restimulated T cells. In B6/*lpr*-p21tg T cells, we observed a reduction in p21 transgene expression following secondary stimulation; this indicates that the high p21 transgene levels at the beginning of restimulation might condition cells to a lower proliferative response during the course of the secondary response (Fig. 16E).

We found that although p21 overexpression inhibits *in vivo* hyperproliferation of B6/*lpr* T cells (Fig. 9B), it does not affect *in vivo* proliferation of B6 T cells (Fig. 9A). Concurring with these results, the p21 transgene differentially affected the *in vitro* proliferation of B6/*lpr* and B6 T cells after secondary stimulation. B6 and B6-p21tg T cell cultures showed similar [^{3}H]thymidine uptake 48 h after both pri-

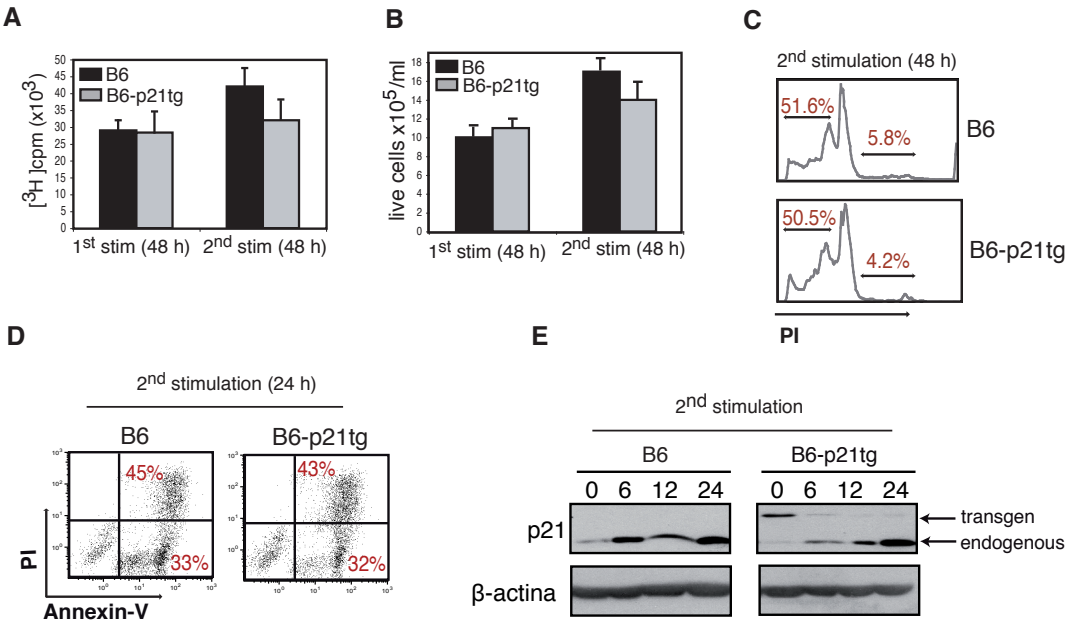


Figure 17. p21 overexpression does not affect proliferation or cell death of control T cells. **A.** Purified CD4+ T cells from B6 and B6-p21tg mice were ConA-stimulated (1st stimulation) or restimulated with ConA after an IL-2 expansion phase (2nd stimulation). Proliferation was measured by [^{3}H]thymidine uptake at 48 h after first or second challenge. Values show mean ± SD (n = 4). **B.** Similar generation of B6 and B6-p21tg CD4+ T cells after primary and secondary stimulation, as determined by Trypan blue dye exclusion. Values show mean ± SD (n = 3). **C.** Cell cycle analysis at 24 h after the second stimulation showed similar proliferation and cell death of B6 and B6-p21tg T cells. Representative histograms are shown (n = 3). **D.** Annexin-V and PI double staining at 24 h after the second stimulation showed the same cell death in B6 and B6-p21tg T cells. Representative histograms are shown (n = 2). **E.** Western blot showing endogenous and transgenic p21 expression in CD4+ T cells from 2 month-old B6 and B6-p21tg mice. Spleen cells were isolated, ConA stimulated, IL-2 expanded and restimulated for indicated times. β-actin was used as a loading control.

mary and secondary challenge (Fig. 17A) and absolute numbers of live cells were similar (Fig. 17B), whereas B6/*lpr*-p21tg T cells showed a reduction in proliferation and live cell numbers (see above). The slightly lower values observed for B6-p21tg compared to B6 T cell cultures after secondary stimulation were not significant.

Cell cycle analysis at 24 h after secondary stimulation of B6 and B6-p21tg T cells demonstrated similar apoptosis levels (hypodiploid cells) as well as comparable proliferation of the two cell groups (Fig. 17C). Double staining with annexin-V and PI showed that a large proportion of B6 and B6-p21tg T cells underwent apoptosis during the first 24 following secondary TCR challenge, and that there were no differences in the proportion of apoptotic cells between the two genotype groups (Fig. 17D); p21 overexpression therefore does not influence B6 T cell responses after secondary stimulation. Western blot analysis confirmed p21 transgene expression in B6-p21tg T cells (Fig. 17E).

Taken together, our *in vivo* and *in vitro* studies suggest that p21 overexpression in control T cells does affect neither proliferative responses nor apoptosis, although it greatly reduces B6/*lpr* T cell hyperproliferation *in vivo* and *in vitro* after secondary challenge. This proliferation-attenuating property of p21 overexpression is not due to a possible apoptosis-inducing effect of the p21 transgene, since both B6/*lpr* and B6/*lpr*-p21tg T cells show the same apoptotic defect. The reduction of B6/*lpr* hyperproliferation is relevant to the outcome of the lupus-like disease, as it ameliorates lymphadenopathy and autoimmune manifestations, even in the severe lupus-like phenotype of MRL-*lpr* mice.

Hyperproliferation of rechallenged B6/*lpr* T cells is associated with NF-κB hyperactivation

The data thus far indicate that Fas plays an important role in the control of T cell proliferation independently of its proapoptotic function. As restimulated B6/*lpr* T cells acquire hyperactivation status and hyperproliferate after secondary stimulation, Fas must have an important function in the control of T cell proliferation after TCR reactivation. Our next objective was to determine the molecular connection between hyperproliferation and hyperactivation in B6/*lpr* T cells.

NF-κB has a fundamental role in regulating the immune response, as a key factor in transcription of the genes essential for cell proliferation and survival. We therefore studied the effect of Fas deficiency on activation of the NF-κB pathway after secondary TCR challenge.

NF-κB dimers are maintained in an inactive state in cytoplasm by NF-κB inhibitor (IκBα). Receptor signaling leads to rapid activation of a multi-subunit kinase

complex (IKK) that phosphorylates IκBα inhibitor on two key serines and marks it for degradation by the ubiquitin pathway (reviewed in Li et al., 2002; Rawilings et al., 2006; Ruland et al., 2003). Although the exact molecular events leading to NF-κB activation are not yet well defined, we know that the CBM complex, formed by CARMA-1, BCL-10 and MALT-1, is essential for NF κB nuclear translocation (Frischbutter et al., 2011; Egawa et al., 2003; Rueda et al., 2005; Sommer et al., 2005; Matsumoto et al., 2005; Thome et al., 2003).

In accordance with the B6/*lpr* T cells hyperproliferation already evident at 24 h post-restimulation, we detected by EMSA analysis that NF-κB in nuclear extracts from B6/*lpr* T cells showed much greater binding to the DNA consensus sequence than that in control extracts (Fig. 18A). In order to confirm and quantify the observed difference, we used the IN Cell Analyzer 2000, a high-content analysis system that quantities data based on automated cell imaging. The integrated software localizes nuclei based on fluorescence staining, and the algorithm defines a collar space around the nuclei. Signal intensity for NF-κB (p65) is then measured in the nuclei and in the peri-nuclear space, and data are expressed as the ratio between nuclear and cytoplasm staining intensity. In correlation with EMSA results, we detected a two-fold increase in NF-κB activation in B6/*lpr* compared to control T cells at 24 h post-secondary stimulation (Fig. 18B, left). We also tested NF-κB activation status in both cell groups 24 h after first stimulation and detected no difference (Fig. 18B, right). These results indicated

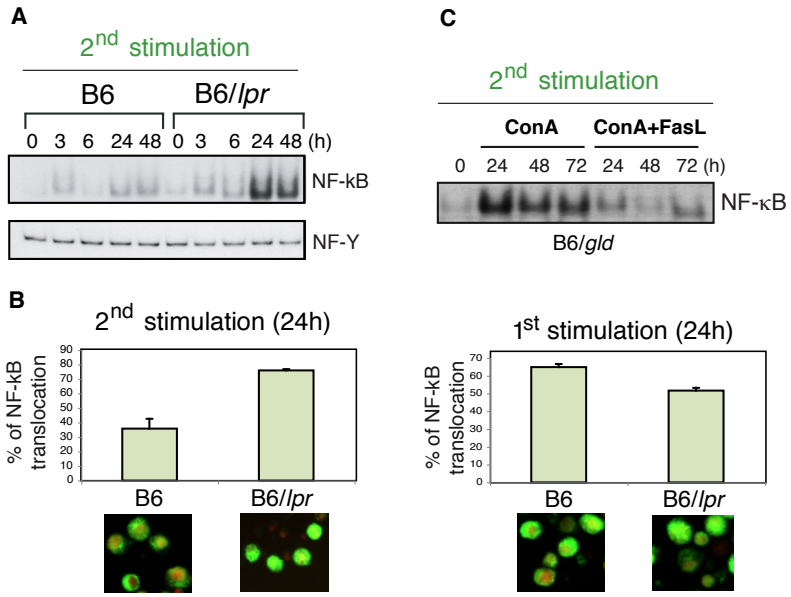


Figure 18. Hyperproliferation of rechallenged B6/*lpr* T cells is linked to NF-κB hyperactivation
A. Enhanced NF-κB activation after secondary stimulation in B6/*lpr* T cells compared to controls, as determined by EMSA. Probe containing the NF-Y consensus sequence was used as a loading control. **B.** Quantification of NF-κB activation levels in B6 and B6/*lpr* T cells using the IN Cell Analyzer 2000 imaging system. Cells at indicated times after second (left) and first (right) stimulation were stained for nuclear content (AAD nuclear stain) and NF-κB (p65). NF-κB signal intensity was measured in nuclei and the perinuclear space, and NF-κB activation status was defined by its nuclear *vs*. cytoplasmic localization. Values show mean ± SD (n = 3). **C.** Reduced NF-κB activation after FasL treatment of B6/*gld* T cells during secondary stimulation in the presence of zVAD (50 μM), as determined by EMSA.

that the hyperproliferation of restimulated B6/*lpr* T cells is a consequence of NF-κB hyperactivation.

In another approach we further confirmed the functional relationship between Fas deficiency and hyperactivation of NF-κB signaling; we treated reactivated B6/*gld* T cells with recombinant FasL (150 ng/ml) in the presence of zVAD (50 μM). Treatment of these FasL-deficient, Fas-sufficient T cells with rFasL restored the Fas signaling pathway and thus led to a decrease in NF-κB activation, as detected by EMSA experiment (Fig. 18C).

NF-κB hyperactivation in B6/*lpr* T cells is associated with caspase-8 whole form activity

Our results established an important role for Fas in T cell homeostasis. We found that in the absence of Fas, B6/*lpr* T cells hyperproliferate after secondary stimulation and that this hyperproliferation is due to NF-κB hyperactivation. In order to obtain insights into molecular mechanism that leads to NF-κB hyperactivation in the absence of Fas, we studied molecular pathway involved in the NF-κB signaling.

It is now very clear that, in addition to promoting apoptosis, active casapse-8 is essential for T cell activation and proliferation after TCR stimulation and its activity is important for NF-κB activation (Chun et al., 2002; Salmena et al., 2003; Siegel 2006; Lamkanfi et al., 2007). The molecular events that lead to caspase-8 activation and its cell localization in these divergent processes remain nonetheless unclear. Some indications support a model in which the location of active caspase-8 would greatly influence its functional capacity as a regulator of cell death or proliferative response (Koenig et al., 2008). It was suggested that caspase-8 translocation into the lipid raft fraction at the plasma membrane is important for T cell activation, and possibly for preventing active caspase-8 from accessing substrates that promote cell death (Mistra et al., 2007).

Given the importance of its spatial location in the mediation of cell growth *versus* cell death, we studied caspase-8 localization in B6 and B6/*lpr* T cells following TCR restimulation. Confocal microscopy was used to visualize caspase-8 and Fas in cells treated with zVAD to block apoptosis. We observed that at time 0, caspase-8 localized mainly to the plasma membrane in both cell groups (Fig. 19A). At 1 h after secondary stimulation, however, caspase-8 staining was almost entirely cytoplasmic in B6 compared to B6/*lpr* T cells; in the latter, its staining was basically annular, indicating that there was no translocation into cytoplasm (Fig. 19B). In addition, caspase-8 and Fas co-localized in control T cells before and after restimulation. We excluded, however, the possibility that this interaction was pro-apoptotic, since all experiments were performed in the presence of the apoptosis inhibitor

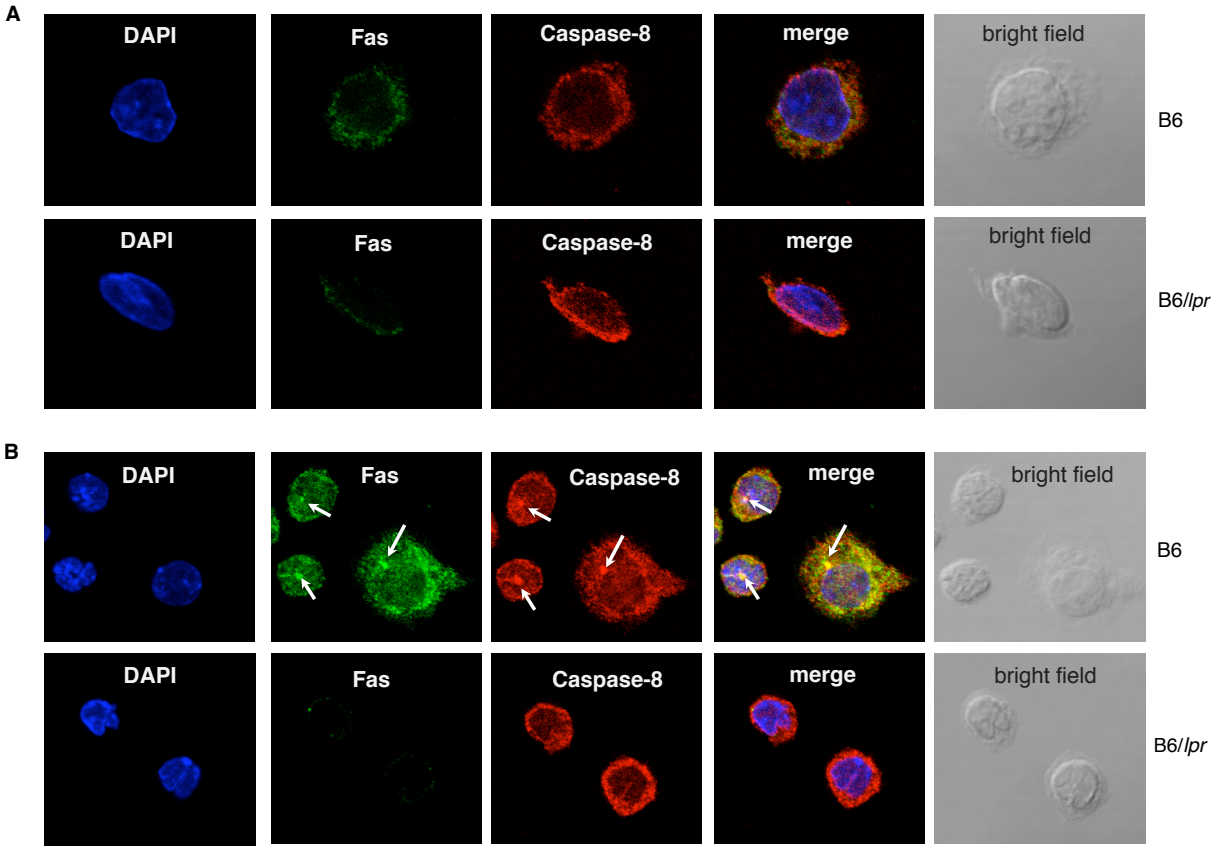


Figure 19. Caspase-8 colocalizes with Fas in B6 T cells before and after restimulation
A. Confocal analysis of Fas and caspase-8 cell localization before ConA restimulation. Cells in the IL-2 expansion phase were collected on collagen-I-coated chamber slides, fixed and permabilized, blocked, and stained with primary antibodies. After washing, cells were stained with secondary antibodies to detect Fas (green) and caspase-8 (red) and mounted with DAPI (blue)-containing mounting medium for confocal microscopy. **B.** Cells after IL-2 expansion were ConA restimulated for 1 h and treated as in (A) for confocal microscopy analysis. Representative results are shown (n = 2).

zVAD. It is thus tempting to speculate that Fas collaborates with caspase-8 in the control of T cell proliferation, possibly by mediating its translocation from the plasma membrane, where the protein complex required for TCR-mediated NF-κB activation, which includes CARMA-1, BCL10 and MALT-1, is located.

Our results show that caspase-8 localizes to distinct cell regions after TCR restimulation in Fas-deficient T cells compared to controls. This suggests that Fas is required for internalization of the pro-proliferative portion of capsase-8.

Although caspase-8 participation in cell activation is well documented, the precise molecular mechanisms that link its activation to TCR-induced proliferation are not yet defined. Following TCR activation, caspase-8 is recruited to the CBM complex (CARMA-1, BCL10 and MALT-1) (Schme 4, Introduction); in this molecular context, caspase-8 contributes to degradation of the NF- κB inhibitor

IκBα and subsequent translocation of NF-κB dimers to the nucleus (Su et al., 2005; Bidere et al., 2006). It was also established that in caspase-8-dependent NF-κB activation, caspase-8 does not require cleavage and dimerization to be active but its enzymatic activity is associated with full-length form (Lamkanfi et al., 2007; Bidere et al., 2006a).

As active unprocessed caspase-8 controls T cell activation and proliferation after primary TCR stimulation, and its activity is important for NF-κB activation, we considered that it might also be involved in hyperproliferation of restimulated B6/*lpr* T cells. To study the caspase-8 activation stage after secondary stimulation, we used biotin-VAD-fmk, which binds selectively to enzymatically active site of the whole caspase molecule. Caspase-8 was precipitated from T cells cultured with zVAD, which assures apoptosis-free conditions. zVAD treatment (50 μM) had

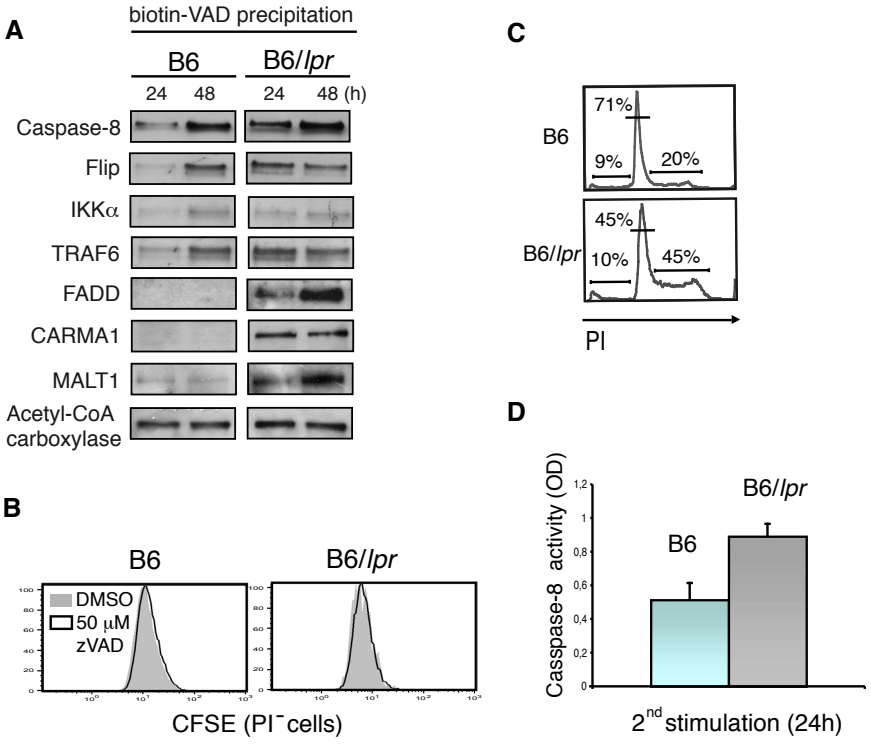


Figure 20. Active unprocessed caspase-8 controls the proliferative response of restimulated T cells independently of its pro-apoptotic function. **A.** Active caspase-8 in its full-length form was isolated from B6 and B6/*lpr* T cells at indicated times after secondary stimulation, using bVAD treatment and precipitation with streptavidin beads, followed by Western blot for caspase-8 and coprecipitated molecules. Endogenously biotinylated acetyl-CoA carboxylase served as a loading control. ConA restimulation was performed in the presence of zVAD (50 μM). Representative data are shown (n = 3). **B.** zVAD treatment had no effect on the proliferation of restimulated B6 and B6/*lpr* T cells, as determined by CFSE dilution. **C.** Cell cycle analysis 24 h post-secondary stimulation of B6 and B6/*lpr* T cells used for bVAD precipitation of active caspase-8. **D.** B6/*lpr* T cells showed higher caspase-8 enzymatic activity 24 h after secondary stimulation, as determined in a colorimetric assay with IEHD-pNA substrate. Release of the pNA group, which indicates relative caspase-8 activity, was measured by spectrophotometry. Values show mean ± SD (n = 3).

no effect on proliferation (Fig. 20B), but efficiently inhibited apoptosis in control T cells (Fig. 20C). Western blot analysis showed that consistent with B6/*lpr* T cell hyperproliferation at 24 h post-secondary stimulation, we detected major caspase-8 activation in these cells (Fig. 20A). We also analyzed coimunoprecipitated proteins, including members of CBM complex and others that were previously showed to be associated with active caspase-8. The composition of the precipitated complex differed for both cell groups; the pro-proliferative protein Flip was associated with the B6/*lpr* T cell complex at 24 h, whereas it was only detected in control T cells at 48 h. This was also the case for IKK complex member IKKα and for the ubiquitin signal transducer TRAF6. FADD, which is essential for proliferative signaling in T cells (Newton et al., 1998; Zhang et al., 1998; Newton et al., 2001), was found exclusively in B6/*lpr* T cells. CARMA1, the CBM complex member that plays a central role in NF-κB signaling (Blonska et al., 2009), was uniquely detected in Fas deficient T cells. Another CBM complex protein, MALT1, was detected in both control and B6/*lpr* T cells, but by 48 h post-stimulation, larger amounts were observed in the B6/*lpr* T cell precipitates. The results indicate major activation of full-length caspase-8 and CBM complex in B6/*lpr* T cells compared to controls after secondary stimulation. Moreover, the active caspase-8 complex tends to assemble lymphocyte proliferation-related signaling molecules more efficiently in B6/*lpr* T cells. Our data suggest that active, unprocessed caspase-8 controls CBM complex activation and proliferation of restimulated B6 and B6/*lpr* T cells independently of its pro-apoptotic function. This justifies NF-κB hyperactivation in Fas deficient T cells.

We further confirmed that caspase-8 was enzymatically active and we assessed this activity by applying a colorimetric assay with IETD-pNA substrate, which has high affinity for caspase-8 (see Methods). We indeed detected higher caspase-8 enzymatic activity 24 h after second stimulation in B6/*lpr* T cells than in controls (Fig. 20D). This data is consistent with B6/*lpr* T cell hyperproliferation. As Fas expression is defective in these cells, it is evident that the caspase-8 activity needed for T cell proliferation is independent of cell death signaling mediated through Fas.

Inhibition of caspase-8 activity attenuates hyperproliferation in rechallenged B6/*lpr* T cells

Inhibition of caspase-8 activity *in vivo* and *in vitro* in human and murine CD4+ T cells leads to reduced proliferation and IL-2 production after primary TCR stimulation (Kennedy et al., 1999; Falk et al., 2004; Mistra et al., 2005), making caspase-8 indispensable for normal lymphocyte proliferation.

In order to demonstrate the relevance

of enhanced caspase-8 activity in Fas deficiency-mediated hyperproliferation of restimulated T cells, we inhibited caspase-8 activity using high zVAD concentrations. At 200 μM, zVAD blocked caspase-8 enzymatic activity in both B6/*lpr* and control T cells, as assessed by colorimetric assay (Fig. 21A). Addition of the inhibitor to cell culture also decreased the amount of bVAD-precipitated caspase-8 in B6/*lpr* T cells (Fig. 21B). We analyzed NF-κB activation after B6/*lpr* T cell restimulation in

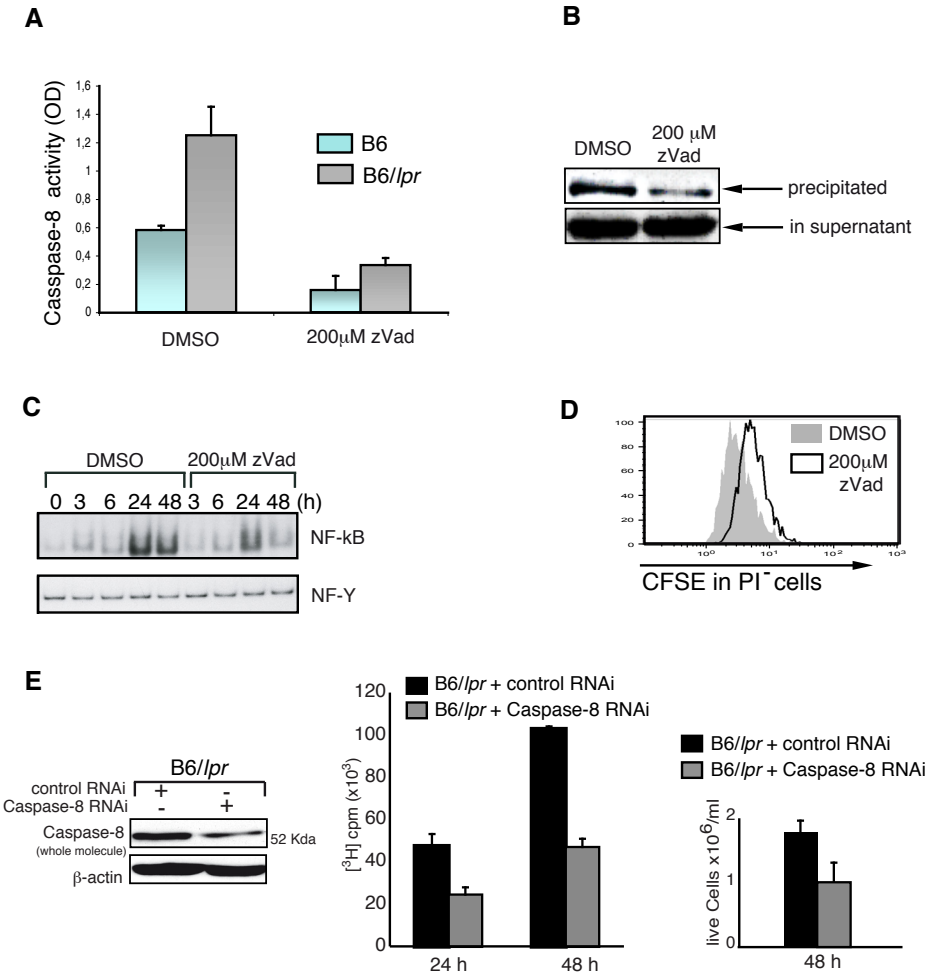


Figure 21. Inhibition of caspase-8 activity attenuates hyperproliferation of rechallenged B6/*lpr* T cells. **A.** Caspase-8 enzymatic activity in B6 and B6/*lpr* T cells determined 24 h after ConA rechallenge by measuring fluorescence release from the cleaved pNA group in the presence of DMSO or zVAD (200 μM). Values show mean ± SD (n = 4). **B.** bVAD precipitation of active caspase-8 from B6/*lpr* T cells 24 h post-ConA restimulation in the presence of DMSO or zVAD (200 μM). **C.** NF-κB activation in B6/*lpr* T cells is reduced by treatment with 200 μM zVAD, as assessed by EMSA. Binding of an NF-Y probe was used as loading control. **D.** Fluorescence intensity dilution of PI-negative CFSE-labeled ConA-rechallenged B6/*lpr* CD4+ T cells, alone or with zVAD (200 μM). Representative data are shown (n = 3). **E.** Caspase-8 protein expression levels from B6/*lpr* T cells transfected with control or capase-8-specific RNAi (left). Reduced proliferation 24 and 48 h after secondary ConA stimulation of B6/*lpr* T cells transfected with control or caspase-8-specific RNAi, determined by [^{3}H]thymidine incorporation (middle). Decrease in the absolute number of B6/*lpr* T cells after transfection with caspase-8-specific RNAi (right). Values show mean ± SD (n = 3).

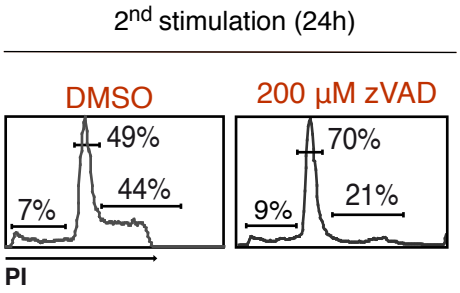


Figure 22. High zVAD concentration (200 μM) does not induce cell viability
Cell cycle analysis 24 h post-secondary stimulation of B6/*lpr* T cells treaded with DMSO or 200 μM zVAD.

the presence of 200 μM zVAD, and found that this caspase-8 activity blockade notably reduced NF-κB activation (Fig. 21C). As predicted, B6/*lpr* T cell hyperproliferation was clearly reduced when zVAD was present, as determined in CFSE dilution experiments, which showed a reduced division rate in CFSE-labeled B6/*lpr* T cells (Fig. 21D), and in cell cycle analysis (Fig. 22B).

These findings confirm that caspase-8 activity is necessary for hyperproliferation of restimulated B6/*lpr* T cells due to its role in NF-κB activation. Our results indicate that in control T cells that survive apoptosis, Fas controls activation and proliferation by negative regulation of caspase-8-dependent NF-κB activation.

Since zVAD treatment can cause autophagy in some cell lines (Yu et al., 2004), 200 μM zVAD might be thought to have adverse effects, including autophagy or toxicity. Nonetheless, single-dose 100 μM zVAD treatment (Alam et al., 1999) and 80 μM zVAD daily for six days (Su et al., 2005) inhibit primary T cell proliferation with no toxic effects or autophagic death. In our assays, we used a single zVAD dose (200 μM) during secondary stimulation of B6/*lpr* T cells; autophagic death was not induced, as double-membrane autophagic vacuoles, abundant in autophagic cells (Galluzzi et al., 2007), were not detected (not shown). Additional experiments excluded zVAD toxicity, since both zVAD (200 μM) and DMSO treatment resulted in normal cell cycle profiles with low proportions of dead cells and no apoptosis induction (Fig. 22).

To further validate the observation that caspase-8 inhibition leads to reduced B6/*lpr* T cell hyperproliferation after rechallenge, we inactivated caspase-8 in these cells by RNA interference (RNAi). Cells were transfected with RNAi ribonucleotides on day 4 of the IL-2 expansion phase, followed by two days in culture with fresh medium and IL-2. Caspase-8 protein levels were reduced in cells treated with caspase-8-specific RNAi, as determined by Western blot (Fig. 21E, left). After secondary ConA stimulation of the cells, we observed that treatment with specific caspase-8 primers substantially reduced proliferation of rechallenged T cells compared to the effect of treatment with nonspecific primers (Fig. 21E, middle), as determined by [^{3}H]thymidine incorporation. The absolute number of live cells was also higher in control RNAi-treated cells than in those treated with caspase-8-specific RNAi, since reduction of caspase-8

levels decreased T cell proliferation after restimulation (Fig. 21E, right). Our data show that reduction of caspase-8 activity inhibits B6/*lpr* T cell hyperproliferation, and implicate caspase-8 in the reported here regulatory function of Fas in the proliferation of apoptosis-surviving T cells. These experiments indicated that inhibition of B6/*lpr* T cell hyperproliferation by targeting downstream TCR signaling could be a useful approach for the study of molecular aspects of the hyperproliferative phenotype in B6/*lpr* mice.

Fas deficiency leads to increase in early NF-κB activation response

Our results demonstrated that hyperproliferating B6/*lpr* T cells at 24 h after the secondary stimulation show NF-κB hyperactivation. We also tested activation of the NF-κB signaling pathway at early times points, since phosphorylation events that lead to the NF-κB response occur shortly after TCR stimulation. We detected higher phosphorylation of IκBα inhibitor in B6/*lpr* T cells than in controls, accompanied by its rapid degradation (Fig. 23A, top). In addition, we analyzed

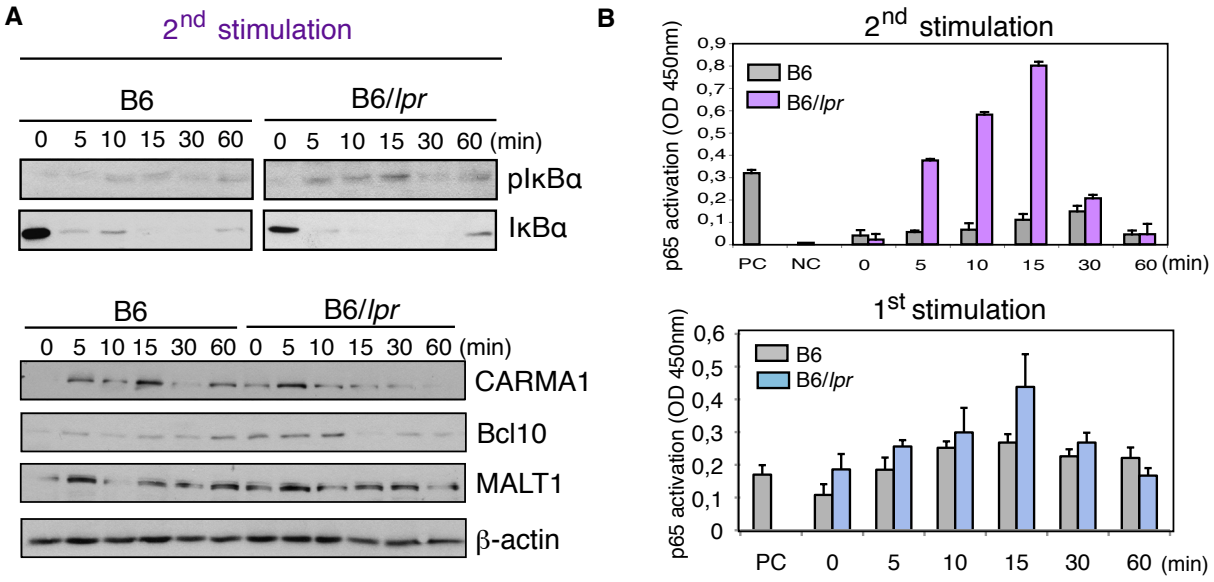


Figure 23. An early NF-κB activation response in B6/*lpr* T cells. A. Western blot shows expression patterns of proteins involved in cell signaling upstream of NF-κB activation after secondary stimulation. β-actin served as a loading control. **B.** NF-κB activation levels in B6 and B6/*lpr* T cells, measured with an ELISA-based kit for NF-κB activation. Detection is based on binding of activated NF-κB homo- and heterodimers from nuclear extracts obtained at indicated times after second (top) and first (bottom) stimulation to the immobilized NF-κB consensus binding site. Jurkat cell nuclear extracts served as a positive controls; wild-type consensus oligonucleotide was used as a negative control. Values show mean ± SD (n = 4).

the expression pattern of the CBM complex proteins; CARMA-1 was expressed earlier in B6/*lpr* T cells than in controls. The magnitude and duration of BCL-10 and MALT-1 expression was also enhanced in B6/*lpr* T cells (Fig. 23A, bottom).

The early NF-κB activation response after the first and the second stimulation was quantified using an ELISA-based kit to detect nuclear factor activation. In this assay, activated NF-κB dimers in nuclear extracts bind the immobilized NF-κB consensus binding site; these complexes are then detected with anti-p65 primary antibody and horseradish peroxidase-conjugated secondary antibody, allowing simple quantification by spectrometry colorimetric readout. In accordance with higher IκBα phosphorylation levels in B6/*lpr* T cells compared to controls, we detected markedly elevated NF-κB activation in nuclear extracts from B6/*lpr* T cells at 5, 10 and 15 min post-restimulation (Fig. 23B, top). The nuclear extract from Jurkat cells activated with TPA (12-O-tetrade- canoylphorbol-13-acetate) and calcium ionophore was used as a positive control for NF-κB p65 activation. As a negative control, wild-type consensus oligonucleotide was used as a competitor for NF-κB binding. This competition assay confirmed that protein subunits that bind to the substrate are specific for the NF-κB consensus binding side. Using the same technique, we also analyzed rapid NF-κB activation after first stimulation, but detected no clear differences (Fig. 23, bottom).

These results show that there is early NF-κB hyperactivation in B6/*lpr* T cells after restimulation, and as these cells continue to hyperproliferate, this hyperactivation is maintained and ultimately support a regulatory role for Fas in the proliferation of apoptosis-surviving T cells.

Fas deficiency affects TCR signaling pathways

The results thus far indicated that Fas deficiency leads to hyperproliferation of restimulated B6/*lpr* T cells. This proliferative advantage was associated with NF-κB hyperactivation, elevated expression of the activation markers CD25 and CD69, and enhanced IL-2 production. All of these cellular responses have a positive effect on cell activation and proliferation; we therefore tested whether ERK1/2 signaling, also known for transduction of proliferative and survival signals, is affected in the absence of Fas during secondary ConA stimulation. Western blot analysis showed that phosphorylation of ERK-1 and ERK-2 was indeed altered in B6/*lpr* T cells following second stimulation (Fig. 24A). These results thus imply that lack of Fas in restimulated T cells permits greater activation of the NF-κB and ERK1/2 signaling pathways, in turn enhancing the proliferative response of B6/*lpr* T cells.

As elevated proliferation of B6/*lpr* T cells relies on signaling pathways downstream of the TCR, it is possible that the TCR triggering is necessary for hyperproliferation. To address this question we subjected the cells to "spontaneous proliferation" assay in which cells were expanded in IL-2, washed, resuspended in fresh culture medium without addition of IL-2 or ConA, and their proliferation rate was tested by PI staining and cell cycle analysis. Proliferation of B6/*lpr* T cells in these conditions was no longer higher than that of control T cells (Fig. 24B). Proliferation of both B6/*lpr* and B6 T cells decreased gradually and cell death increased, reaching an apoptotic peak at 12 h. In an analysis of cell surface expression of CD25, CD69 and FasL, we confirmed that this cell death was not AICD, but rather a cell response to nutrients and IL-2 withdrawal (not shown); we therefore concluded that B6/*lpr* T cell hyperproliferation requires a TCR-mediated activation. Absence of Fas during restimulation thus appears to potentiate TCR signaling pathways necessary for T cell proliferation and IL-2 production, through its capacity to augment the NF-κB and ERK1/2 signaling pathways.

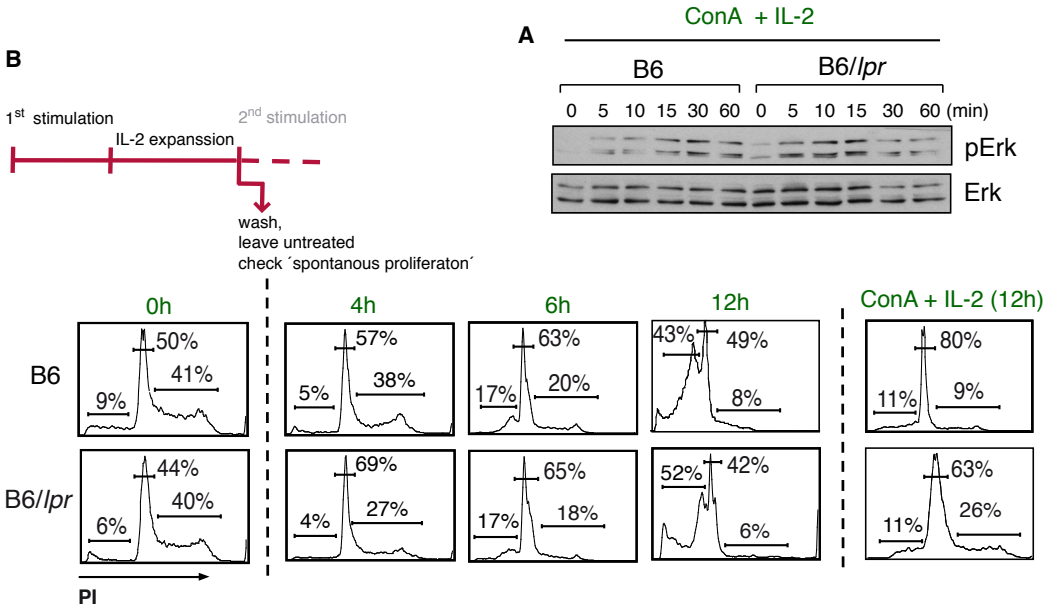


Figure 24. TCR dependency of the B6/*lpr* hyperproliferative phenotype
A. Western blot shows ERK1/2 phosphorylation at different times after secondary stimulation in B6 and B6/*lpr* T cells. **B.** Hyperproliferation of B6/*lpr* T cells after secondary stimulation is a TCR-mediated phenomenon. Cell cycle and apoptosis analysis of B6 and B6/*lpr* T cells, ConA restimulated, or washed and left untreated. Representative histograms are shown (n = 6).

Fas afects TCR proximal signaling events to control T cell proliferation

To gain more information about the nature of B6/*lpr* T cell hyperproliferation and its relationship to TCR-dependent signaling, we explored the emplacement of the Fas inhibitory action in T cell proliferation. We used PMA and ionomycin, stimuli that bypass TCR engagement but trigger normal T cell proliferative responses. In this context, PMA acts in the same manner as diacyglycerol (DAG) in cell stimulation through the TCR and activate protein kinase C (PKC); ionomycin induces Ca2+/calmodulin-dependent signaling pathways and synergizes with PMA to enhance PKC activation (Chatila et al., 1989). This stimulation induces a high proliferative response, accompanied by IL-2R expression and IL-2 synthesis.

Cell cycle analysis showed that in contrast to TCR triggering-induced proliferation, the same Fas-deficient CD4+ T cells proliferated normally in response to stimulation with PMA + ionomycin (0.5 μg/ml and 20 ng/ml, respectively; Fig. 25A, top). Their proliferation in response to stimulation with ConA + IL-2 (3 μg/ml and 20 ng/ml, respectively) or with anti-CD3/CD28 (0.5 μg/ml and 1 μg/ml, respectively) was clearly enhanced compared to controls (Fig. 25A, middle and bottom). These results indicate that Fas acts upstream or at the level of PKC and/or Ca2+ signaling in the TCR proliferative pathway. Consistent with these data, ionomycin and PMA induced an increase in free intracellular Ca2+ concentration in B6/*lpr* T cells, which was nonetheless lower than that induced in control T cells (Fig. 25B, top). TCR stimulation, which in this case was with anti-CD3/CD28, lead to an enhanced Ca2+ increase in the absence of Fas, indicating that Fas participates in TCR proximal signaling events (Fig. 25B, bottom). It is of interest to note that T cells from SLE patients can regain normal levels of proliferation when stimulated with PMA (Sierakowski et al., 1989), suggesting that signaling defects in these cells might also reside in the proximal signal transduction molecules upstream of PKC (Dayal et al., 1991).

To further characterize the activation defects of Fas-deficient T cells, we analyzed several TCR-induced signaling events. After secondary stimulation, the magnitude and duration of TCR expression was consistently elevated in B6/*lpr* compared to control T cells. Enhanced phosphorylation of Src (sarcoma tyrosine kinase) on residue Tyr416, ZAP-70 (ζ chain-associated protein kinase 70 kDa), LAT (linker for activation of T cells) and PKCα (α protein kinase C) (Fig. 25C) was also evident in B6/*lpr* T cells, suggesting that Fas deficiency influences early signaling steps in the proliferative pathway. Interestingly, we detected increased phosphorylation of these proteins at time 0 in B6/*lpr* T cells, which suggests that these cells

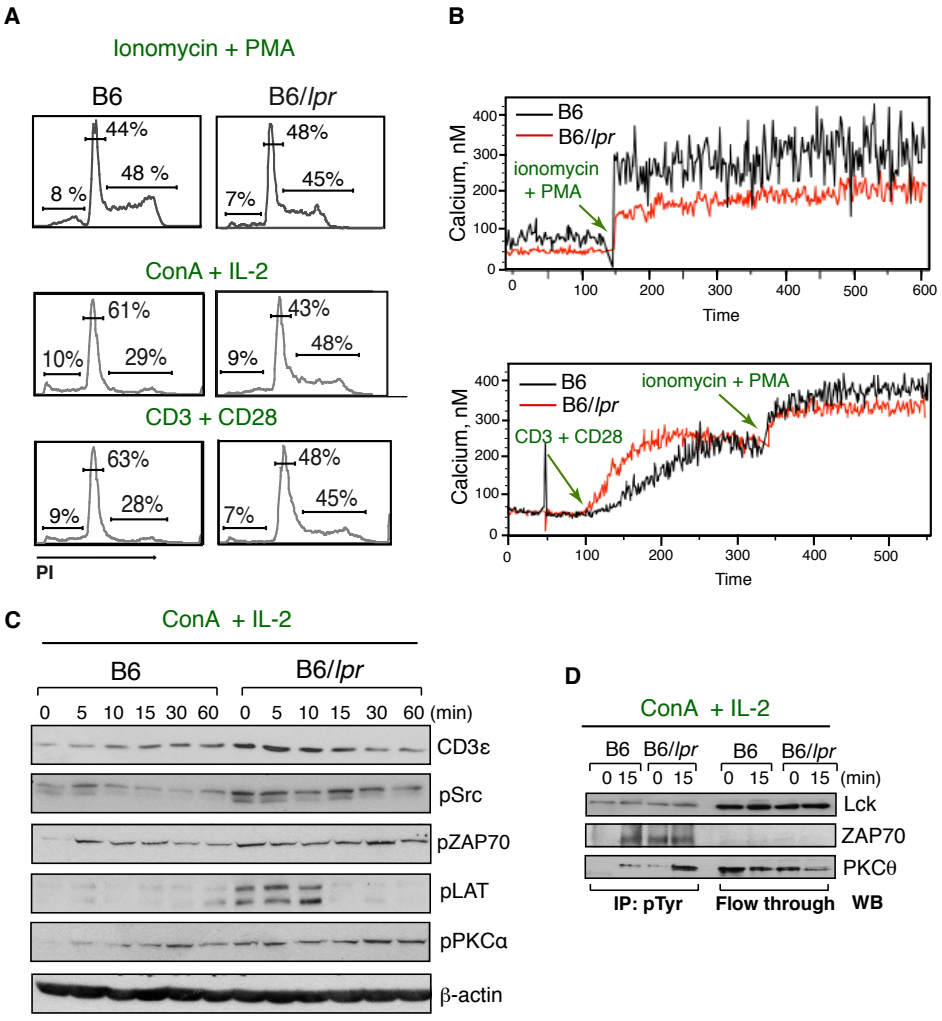


Figure 25. Fas interacts with TCR proximal signaling events to control T cell proliferation. **A.** After primary stimulation and IL-2 expansion, control and B6/*lpr* T cells were restimulated with ionomycin (0.5 μg/ml) and PMA (20 ng/ml), with ConA (3 μg/ml) and Il-2 (20 ng/ml), or with anti-CD3 (0.5 μg/ml) and anti-CD28 (1 μg/ml), and PI-stained for cell cycle analysis. Representative histograms are shown (n = 3). **B.** Calcium flux was measured by flow cytometry with Fluo-2-loaded B6 and B6/*lpr* T cells. Basal fluorescence readings were taken for 100 s before restimulation with ionomycin (0.5 μg/ml) and PMA (20 ng/ml), or with anti CD3 (5 μg/ml) and -CD28 (1 μg/ml) followed by ionomycin/PMA (doses as above). Representative results are shown (n = 3). **C.** Western blot showing the kinetics of TCRε expression and phosphorylation pattern of proteins involved in early T cell proliferative signaling. Cell lysates were obtained from ConA-restimulated B6 and B6/*lpr* T cells at indicated times. β-actin served as a loading control. **D.** Immunoprecipitation of tyrosine-phosphorylated proteins after restimulation with ConA and IL-2 of B6 and B6/*lpr* T cells; immunoblotting with antibodies specific for indicated proteins indicates their tyrosine phosphorylation levels or their association with tyrosine-phosphorylated proteins at time 0 and 15 min post-restimulation.

were already in the pre-activation stage. This activation status has no functional significance, however, since we detected no NF-κB activation at time 0 (Fig. 18A) and only TCR-restimulated T cells hyperproliferate (Fig. 24B).

We further characterized early signaling events in the absence of Fas by

immunoprecipitating phosphorylated tyrosines from B6/*lpr* and B6 T cells at time 0 and 15 min post-restimulation. Western blot with Lck-, ZAP70- and PKCθ-specific antibodies showed higher levels of these tyrosine-phosphorylated proteins in B6/*lpr* T cells (Fig. 25D); again the difference was especially obvious at time 0.

In summary, our findings implicate Fas as a regulator of proximal TCR signaling events. These results further strengthen our view that in addition to inducing apoptosis Fas has another fundamental role in the maintenance of T cell homeostasis through negative control of early events that lead to activation of proliferative pathways such as NF-κB and ERK1/2.

Fas deficiency affects TCR downregulation after second stimulation

B6/*lpr* T cells hyperproliferate following restimulation, and this process depends on signaling pathways downstream of TCR (NF-κB, ERK1/2). We thus considered that analysis of TCR expressed on these cells at different stages of the AICD protocol could help to clarify the mechanism underlying hyperproliferation, and possibly the pathogenesis of autoimmune disease in B6/*lpr* mice. We stained cells with anti-TCR antibody, followed by flow cytometry analysis, and observed a slight decrease in B6/*lpr* TCR expression following the first stimulation; TCR expression equalized between the two cell groups during the IL-2 expansion phase. After the second stimulation, cell surface TCR expression in B6/*lpr* T cells was not downregulated as it was in B6 T cells (Fig. 26A, top). The dynamics of surface TCR expression in both T cell groups is better expressed in Fig. 26A (bottom), showing that after rechallenge, surface TCR expression was downregulated in control T cells and was lower than during the IL-2 expansion phase. This downregulation could allow control T cells to terminate signaling and thus to shut down the proliferative response. Indeed, TCR/CD3 downmodulation is suggested to be one of the mechanisms that contribute to the signaling arrest initiated by antigen binding (Valitutti et al., 1996; Valitutti et al., 1997; Cai et al., 1997; Liu et al., 2000; Davanture et al., 2003). Persistent TCR expression on B6/*lpr* T cells could otherwise account for enhanced, prolonged signaling, whose lack of termination could lead to the hyperproliferative phenotype observed in these cells.

To confirm that this TCR downmodulation defect in B6/*lpr* T cells was due to the Fas deficiency and due to the lack of interaction with FasL, we analyzed surface TCR expression in B6/*gld* T cells in which Fas-FasL signaling was restored by addition of rFasL (150 ng/ml). Treatment of B6/*gld* T cells with FasL in the presence of zVAD (50 μM, to avoid FasL-induced apoptosis) lowered surface TCR expression as soon as 1 h after second stimulation (Fig. 26B).

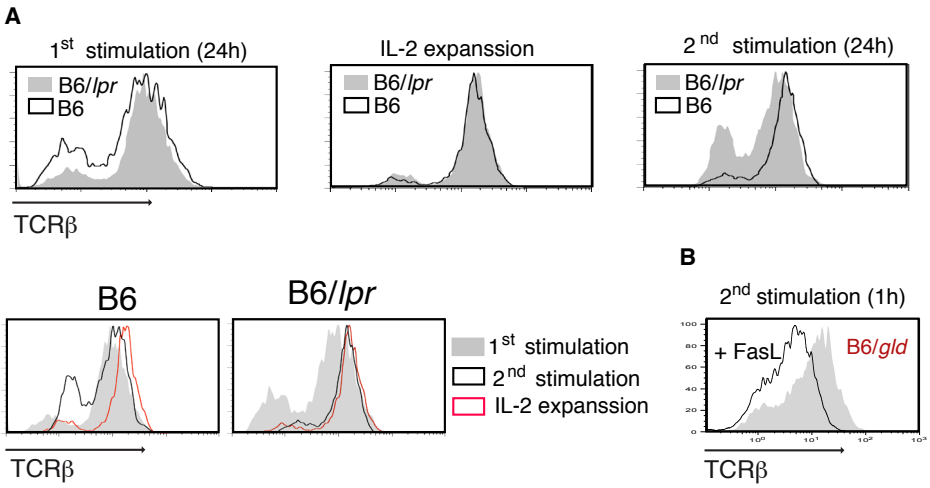


Figure 26. Fas deficiency affects TCR downregulation after secondary stimulation
A. Cells at indicated stages in the AICD protocol were stained with anti-TCRβ antibody, and surface TCR expression was compared between B6 and B6/*lpr* T cells (top) and between different protocol stages for each genotype (bottom). Representative data are shown (n = 3 mice/genoptype). **B.** FasL treatment (150 mM) of B6/*gld* T cells at the time of ConA restimulation reduced surface TCRβ expression after 1 h. Representative data are shown (n = 4).

TCR internalization is impaired in B6/*lpr* T cells after secondary stimulation

Our data thus indicate that after secondary stimulation in the absence of Fas, cells show a greater proliferative capacity that is associated with enhanced cell signaling downstream of the TCR, resulting from a Fas deficiency effect on TCR expression. The absence of Fas thus impairs TCR downmodulation, which can result in prolonged signaling that can in turn provoke a hyperproliferative phenotype in B6/*lpr* T cells.

We found that B6/*lpr* T cells do not downregulate their TCR by 24 h post-restimulation, which prompted us to speculate that prolonged signal transduction from the T cell surface could be the cause of elevated proliferation of these cells. To better understand the nature of this TCR downmodulation defect in B6/*lpr* T cells, we stimulated B6 and B6/*lpr* T cells with ConA + IL-2 in the presence of zVAD (50 μM) and analyzed surface TCR expression by flow cytometry. Shortly after restimulation (3 h), the cell surface TCR level was sharply

downregulated in B6 T cells, which was not the case for B6/*lpr* T cells (Fig. 27A). The apoptosis blockade with zVAD ruled out the possibility that the decrease in TCR expression in control T cells was due to cell death. This suggests that TCR downregulation in normal T cells might be a physiological process that leads to negative regulation of T cell activation.

To determine the kinetics of TCR expression in B6/*lpr* and B6 T cells, we stimulated these cells for the indicated times and analyzed TCR expression by Western blot. TCR expression in B6/*lpr* T cells was constant and did not follow the kinetics of control TCR, which showed initial upregulation and subsequent downregulation at 1 h post-stimulation (Fig. 27B), in accordance with the downmodulation defect detected by flow cytometry.

We next studied the reason for impaired TCR downregulation in B6/*lpr* T cells. Various cell processes, such as receptor internalization, degradation of an internalized receptor fraction, recycling and production of new receptors, contribute to the final pool of surface receptors. To determine whether Fas deficiency affected TCR internalization, we used a cell surface fluorescence quenching technique in which cells at distinct stages of the AICD protocol were stained with Alexa488-conjugated anti-TCRβ (45 min, on ice). We used the anti-TCRβ H57 clone, which is the only suitable antibody for this approach, as it stably binds the TCR and does not dissociate during internalization (Davanture et al., 2005). After washing off unbound antibody, we pulsed cells at 37°C to trigger internalization, which was terminated by placing cells on ice at various times. Cells from each sample were divided into two groups, one fixed directly with formaldehyde and the other treated with anti-Alexa488 quenching antibody (25 μg/ml) prior to fixing and flow cytometry analysis. We quantified mean fluorescent intensity of all samples and calculated internalized fluorescence as described (Austin et al., 2004). To avoid pH changes, which mediate internalized receptor sorting to distinct endosomal compartments, all internalization assays were performed in CO_2-independent culture medium. The caspase inhibitor zVAD was used in all experiments to equalize death conditions. In accordance with the defective TCR downregulation described above, we detected impaired TCR internalization in B6/*lpr* T cells in response to secondary stimulation (Fig. 27C). In control T cells, rapid receptor internalization began at 10 min, and more than 70% of the TCR had been internalized by 1.5 h. In contrast, B6/*lpr* T cells maintained their initial internalization level (~20%) throughout the assay, with only 30% TCR internalization at 1.5 h. These results indicate impaired TCR internalization in B6/*lpr* T cells; consequently, Fas is necessary for TCR-triggered early signaling events.

We also tested whether Fas affects TCR internalization after primary stimulation. In experiments similar to those described above, we found the same de-

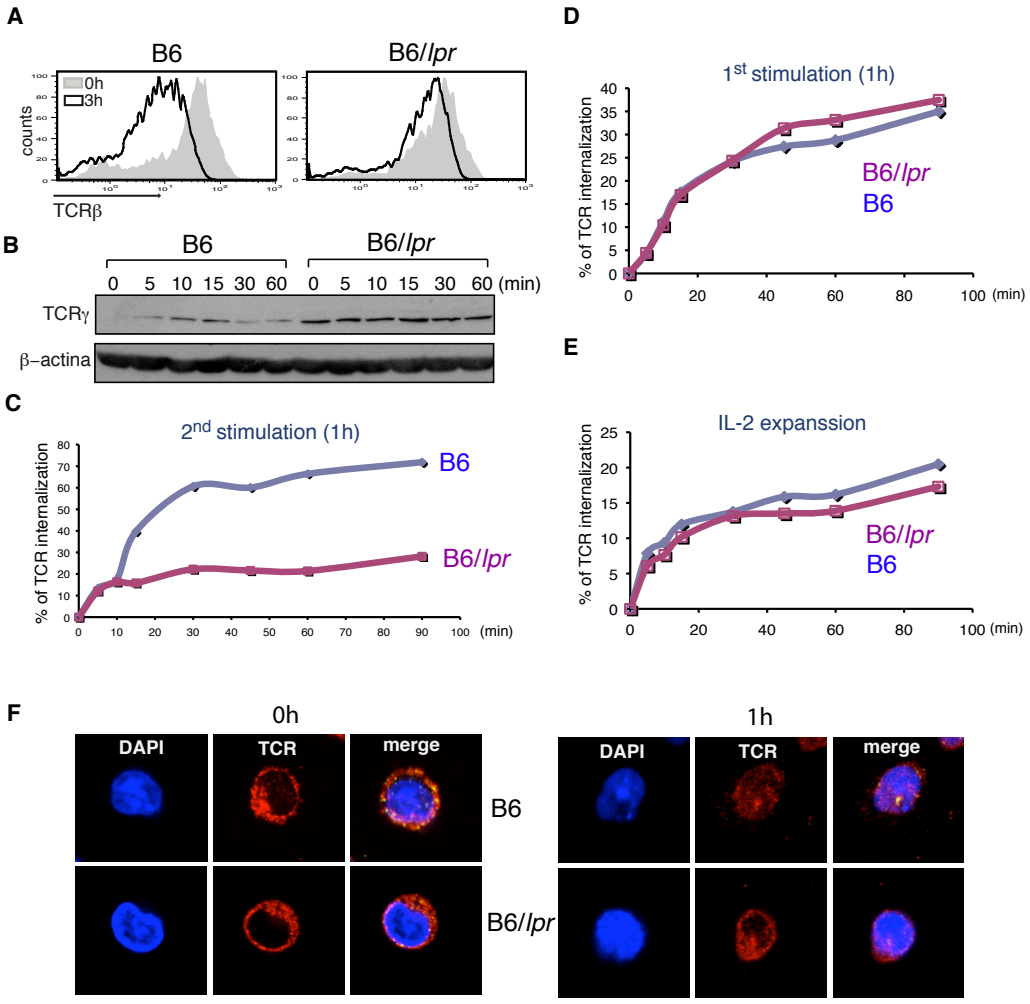


Figure 27. Defective TCR internalization after secondary stimulation in B6/*lpr* T cells
A. The TCR in B6/*lpr* T cells is not downregulated after restimulation, as determined by flow cytometry analysis for surface TCRβ expression. Representative results are shown (n = 5). **B.** Western blot showing kinetics of TCRγ expression in B6 and B6/*lpr* T cells after ConA restimulation for indicated times. β-actin served as a loading control. **C.** TCR internalization ratio in B6 and B6/*lpr* T cells as determined by cell surface fluorescence quenching. Cells at the end of the IL-2 expansion phase were ConA restimulated for 1 h prior to surface staining with Alexa488-anti-TCR antibody, then pulsed at 37°C for indicated times to internalize receptor, washed, and divided into two groups. One group was directly fixed and the other incubated with quenching antibody prior to fixing and FACS analysis. Representative data are shown (n = 4). **D.** Freshly isolated B6 and B6/*lpr* T cells were stimulated for 1 h with ConA and IL-2 (1st stimulation) and TCR internalization was analyzed as in (C). Representative results are shown (n = 3). **E.** Surface fluorescence quenching (as in C) was used to assess the TCR internalization ratio of B6 and B6/*lpr* T cells during the IL-2 expansion phase. Representative data are shown (n = 2). **F.** Confocal analysis of TCR redistribution before and 1 h after ConA restimulation. Cells during IL-2 expansion were collected on collagen-I-coated chamber slides and left untreated (0 h, left) or ConA restimulated (1 h, right). Cells were placed on ice to terminate stimulation-induced internalization, fixed and permabilized, blocked, and stained with primary antibody. After washing, cells were stained with secondary antibody to detect TCR (red) and mounted with DAPI (blue)-containing mounting medium for confocal microscopy. Representative results are shown (n = 2).

gree of TCR internalization in B6/*lpr* and B6 T cells at 1 h after the first stimulation (Fig. 27D). Likewise, we detected no difference in TCR internalization between these two cell groups during the IL-2 expansion phase (Fig. 27E). Fas thus affects TCR internalization only after the second stimulation.

We next used confocal microscopy to explore TCR localization in these cells before and after second stimulation. In both cell groups, TCR staining was distributed across the plasma membrane at time 0 (Fig. 27F, left). At 1 h after restimulation, control T cells showed TCR staining throughout the entire cell, whereas the TCR in B6/*lpr* T cells continued mainly at the plasma membrane (Fig. 27F, right). This result further supports the finding of defective surface TCR downregulation in B6/*lpr* T cells.

TCR internalization is an example of clathrin-mediated endocytosis (Krangel 1987; Minami et al., 1987; Crotzer et al., 2004). Transferrin is a major blood glycoprotein that transports iron to all tissues from the liver and the intestine. All growing cells bear surface transferrin receptors that bind ferrotransferrin (a transferrin form with two bound Fe^{3+} ions). The transferrin receptor mediates transferrin endocytosis through a clathrin-dependent internalization mechanism (Iacopetta et al., 1988; Damke at al., 1994). To test whether defective TCR internalization in B6/*lpr* T cells is specific to this receptor or whether the entire clathrin-mediated endocytosis process is affected by Fas deficiency, we compared the rate and extent of transferrin internalization in B6 and B6/*lpr* T cells after secondary stimulation. In B6 T cells, ~60% of the cell surface transferrin receptor underwent endocytosis by 90 min post-pulse. This value was essentially identical to that for B6/*lpr* T cells (Fig. 28A), indicating that impaired TCR internalization in Fas-deficient T cells is specific to the TCR receptor and not to the general endocytosis pathway. We also performed a transferrin internalization assay at 24 h post-restimulation, and detected no difference in internalization rates between B6 and B6/*lpr* T cells (Fig. 28B). To follow up TCR internalization, we repeated the surface fluorescence quenching assay at 24 h after secondary stimulation, and continued to observe a difference in the TCR internalization rate between the two cell groups (Fig. 28C).

As TCR downmodulation also involves receptor recycling, we further analyzed the nature of the TCR downmodulation defect in B6/*lpr* T cells, by examining the TCR recycling process using the fluorescence quenching technique. In these experiments, cells were preincubated with Alexa488-anti-TCRβ (2 μg/ml, 15 min 37°C), then rapidly chilled on ice, washed and surface-quenched (15 min, on ice) with anti-Alexa488 quenching antibody (25 μg/ml). Cells were pulsed at 37°C in the continuous presence of the quenching agent, then rapidly chilled at different time intervals, fixed, and analyzed by flow cytometry. The percentage of internalized and recycled fluorescence was calculated as the difference between

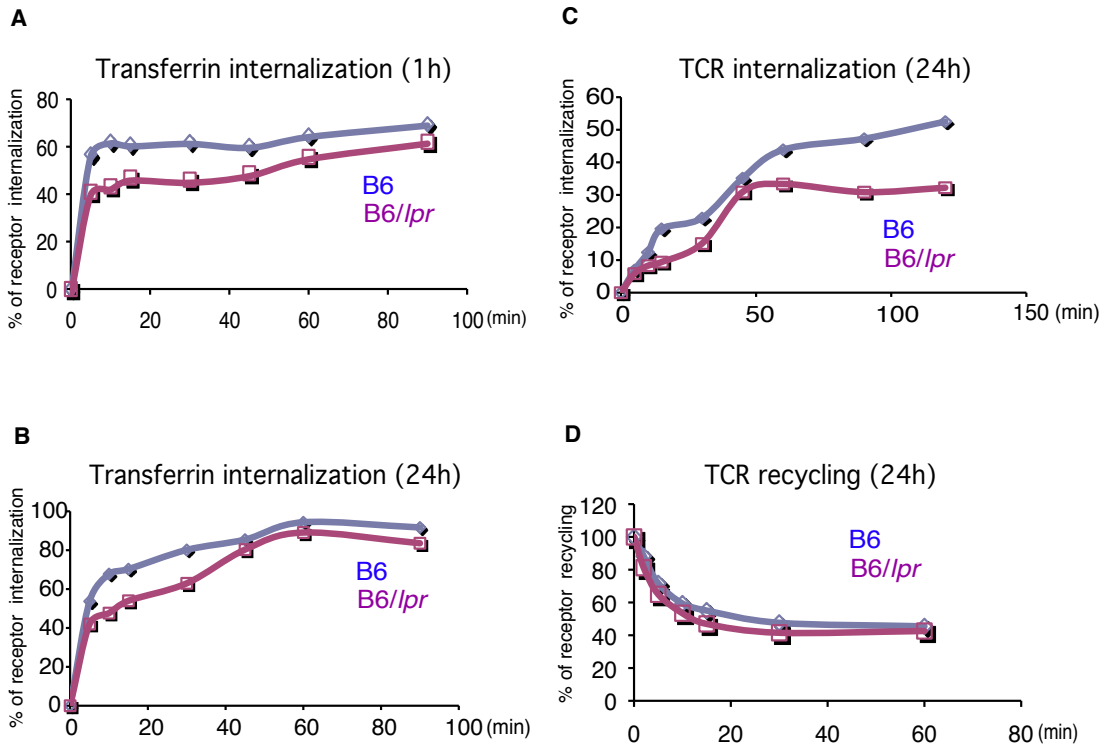


Figure 28. Impaired internalization in Fas-deficient T cells is specific to TCR
A. B6 and B6/*lpr* T cells at the end of the IL-2 expansion phase were ConA restimulated for 1 h to trigger internalization, and cells were incubated at 37°C with Alexa488-transferrin for indicated times. Cells were washed and divided into two groups; one was directly fixed and the other, incubated with quenching antibody prior to fixuing and FACS analysis. Representative data are shown (n = 3). **B.** Transferrin internalization experiment as in (A), in B6 and B6/*lpr* T cells restimulated for 24 h. Representative results are shown (n = 2). **C.** TCR internalization assay (as in Fig. 13C) performed with B6 and B6/*lpr* T cells restimulated for 24 h. Representative data are shown (n = 2). **D.** TCR recycling was measured in B6 and B6/*lpr* T cells restimulated with ConA for 24 h. Cells were stained with Alexa488-anti-TCR at 37°C to internalize labeled receptor, then incubated on ice with quenching anti-Alexa488 antibody, and warmed to 37°C in the continuous presence of the quenching agent for indicated times. Recycling of Alexa488-labeled TCR induced quenching of surface fluorescence. Representative results are shown (n = 3).

pulsed/unquenched fluorescence (time 0) and pulsed/quenched fluorescence at each chase time, and normalized to time 0. We detected no difference in the TCR recycling ratio between B6 and B6/*lpr* T cells (Fig. 28D), which suggests that defective internalization rather than recycling accounts for the lack of TCR downmodulation in B6/*lpr* T cells.

In summary, the data demonstrate that Fas deficiency leads to inhibition of TCR internalization after the second stimulation. Defective TCR internalization in B6/*lpr* T cells might result in prolonged triggering of the signal transduction pathways, leading to enhanced NF-κB and ERK1/2 activation, and finally to T cell hyperactivation and hyperproliferation.

Fas direct interaction with the TCR complex

Our findings indicated that Fas is needed for correct TCR downregulation following secondary stimulation, and that lack of Fas leads to impaired internalization of the TCR. This prompted us to test whether Fas interacts directly with the TCR during a second stimulation, for which we used confocal microscopy localization analysis. Cells were prepared (see Methods) and analyzed for Fas and TCR localization at time 0 and 1 h after secondary stimulation. In control T cells, Fas was distributed over the entire plasma membrane, coinciding with fluorescence staining for the TCR (Fig. 29, top). In B6/*lpr* T cells, TCR was also located at the plasma membrane at time 0; we detected slight Fas staining, as although this protein is defective, it is expressed in trace amounts in these mice (Fig. 29, top). Sequential analysis of Fas and TCR distribution showed that these proteins co-localized in control T cells at 1 h post-restimulation (Fig. 29,

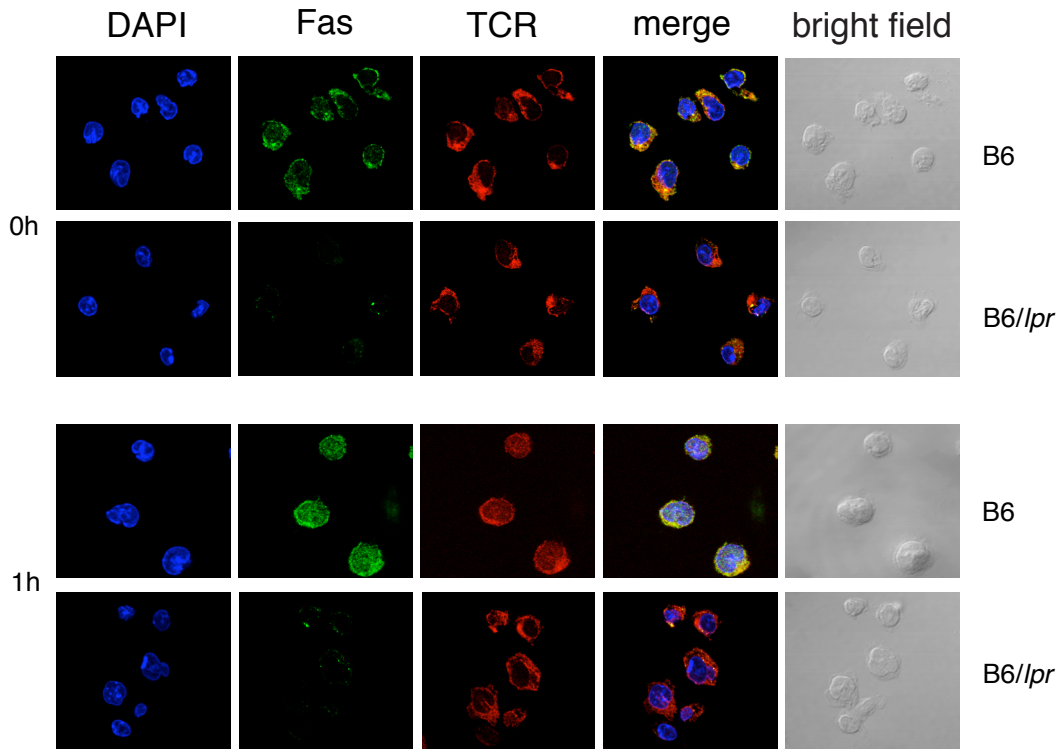


Figure 29. Fas colocalizes with TCR before and after secondary stimulation in control T cells. Confocal analysis of Fas and TCR redistribution before and 1 h after ConA restimulation. Cells in the IL-2 expansion phase were collected on collagen-I-coated chamber slides and left untreated (time 0, top) or ConA restimulated (1 h, bottom). Cells were placed on ice to terminate stimulation-induced internalization, fixed and permabilized, blocked, and stained with primary antibodies. After washing, cells were stained with secondary antibodies to detect Fas (green) and TCR (red) and mounted with DAPI (blue)-containing mounting medium for confocal microscopy. Representative results are shown (n = 3).

bottom). Both proteins translocated from the plasma membrane to the cell interior, which might reflect internalization triggered by antigen binding. In B6/*lpr* T cells at 1 h after secondary stimulation, the TCR remained mostly membrane-associated, in accordance with our previous results (Fig. 27F, right). In conclusion our findings show that Fas co-localizes with the TCR before and after secondary stimulation, which could allow direct Fas influence on TCR internalization and other TCR-dependent signaling events.

The confocal data demonstrated only that Fas and TCR co-localized in control T cells. To assess a possible physical interaction between these two proteins, we performed a TCR immunoprecipitation assay at various times after restimulation in the presence of zVAD. Fas indeed associated with TCR after secondary stimulation in B6 T cells (Fig. 30); this association was transient and peaked at 10-15 min following ConA restimulation, indicating that the two proteins interact directly in the context of repeated stimulation. There were also notable differences in the composition of the immunoprecipitated complex from the two cell types. At difference from control T cells, Lck recruitment was very efficient in B6/*lpr* T cells, and was detected even at time 0. This was also the case for ZAP70, whose association with TCR in B6/*lpr* T cells was constant over the 1 h course of the stimulation. In contrast, ZAP70 recruitment to TCR in control T cells began at 5 min, peaked at 10 min, and decreased by 1 h post-restimulation. PKCθ association with TCR in Fas-deficient T cells resembled that of Lck, with recruitment at time 0 that remained essentially constant throughout the 1 h period after the second stimulation. In control T cells, PKCθ binding to TCR could barely be detected, and only at later time points (30 and 60 min) (Fig. 30).

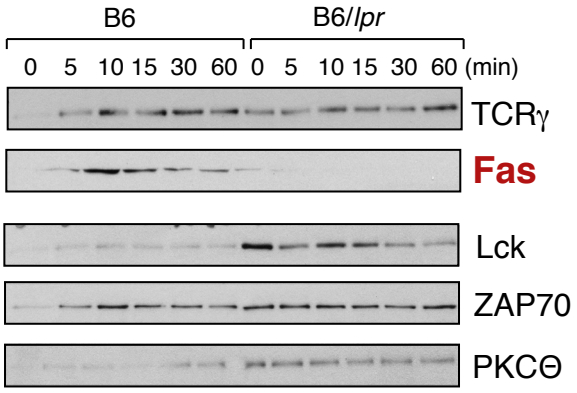


Figure 30. Direct interaction of Fas with TCR signaling complex in control T cells
Fas coimmunoprecipitates with TCR in B6 T cells. B6 and B6/*lpr* T cells were ConA restimulated for the indicated times and immunoprecipitated with anti-TCRγ antibody. Western blot with primary and secondary antibodies showed association of the indicated proteins with the TCR in B6 and B6/*lpr*.

These results indicate that in control T cells Fas is recruited to the TCR complex after restimulation, which might impede aggregation of proteins important in TCR-triggered signaling pathways; lack of this obstruct could otherwise result in hyperactivated proliferative signaling, as is the case in Fas-deficient B6/*lpr* T cells.

Severe deregulation of immune homeostasis in B6/*lpr* mice

Homeostasis regulates total lymphocyte number by balancing cell growth and death. Programmed cell death, referred to as apoptosis, eliminates activated or autoreactive lymphocytes, and is thus important in immune system homeostasis. Interaction of Fas with its ligand (FasL) after secondary TCR triggering *in vitro* induces apoptosis *via* activation-induced cell death (AICD). Fas/FasL interaction recruits the adaptor protein FADD, which activates caspase-8 and initiates the apoptotic cascade. T cell apoptosis is considered central to lymphocyte homeostasis and tolerance induction (Walker and Abbas, 2002); this concept was recently confirmed by the observation of increased lymphadenopathy in Fas/Bim double-deficient mice (Green, 2008).

Fas deficiency has a considerable impact on immune system homeostasis in B6/*lpr* mice. These animals develop an autoimmune disease characterized by lymphadenopathy and splenomegaly, hyperproliferation (Fig. 1D) and accumulation of double negative (DN) T cells, accumulation of CD4+ memory T cells (Fig. 12A), elevated serum levels of anti-DNA antibodies (Fig. 12B), immune complex formation, and glomerulonephritis.

Lack of Fas-triggered apoptosis in activated T cells of B6/*lpr* mice was suggested to be a direct cause of lymphadenopathy development and lupus-like disease manifestations (Watanabe-Fukunaga et al., 1992). The basis of autoimmune disease in these mice nevertheless remains elusive, since the *in vitro* B6/*lpr* T cell apoptotic defect is not always associated with reduced apoptosis *in vivo* (Walker and Abbas, 2002). In addition, T cell death defects do not completely explain the B6/*lpr* phenotype, since in the absence of other components of the apoptotic pathway; lack of apoptosis is not accompanied by lymphadenopathy development. This is the case of transgenic mice that overexpress the caspase-8 inhibitor CrmA in T cells (Smith et al., 1996); although AICD of these T cells was inhibited efficiently *in vitro*, the mice showed no DN T cell accumulation or lymphadenopathy. This observation suggests that in addition to its role in apoptosis, Fas might have another function in homeostasis control. Similarly, mice with caspase-8 deficiency in T cells (Chun et al., 2002; Salmena et al., 2003) and transgenic mice that overexpress a dominant negative form of FADD (Newton et al., 1998; Zhang et al., 1998; Newton et al., 2001) show defective apoptosis, accompanied not by lymphadenopathy development but rather by immunodeficiency. These reports, as well as data from our laboratory, indicate that in addition to its pro-apoptotic role, Fas/FasL signaling has other important cell regulatory functions. Here we

examined whether an apoptosis-independent function of Fas contributes to the homeostasis of activated T cells.

Fas negatively controls proliferation of repeatedly activated T cells

In this study, we established that Fas negatively controls the proliferation of restimulated T cells. Following secondary antigen stimulation, B6/*lpr* T cells hyperproliferated in comparison to controls (Fig. 3A, top). Given the role of Fas as an apoptosis inducer, we focused our efforts on evaluating whether the B6/*lpr* T cell proliferative advantage reflects greater accumulation of apoptosis-resistant T cells or an increase in the proliferative capability. We used the inhibitor zVAD (50 μM) to equalize cell death conditions between Fas-deficient and control T cells, and observed that B6/*lpr* T cells still manifested a higher proliferation ratio than controls (Fig. 3A, bottom). This increased division rate was confirmed by analyzing expression of the proliferation marker Ki-67 (Fig. 3E) and in CFSE dilution experiments (Fig. 3D). These data indicated that enhanced B6/*lpr* T cell proliferation is not a direct result of the apoptosis defect, but a consequence of a greater proliferative capacity, which suggests that Fas negatively controls the expansion of apoptosis-surviving T cells.

Further confirmation that the proliferative differences between control and Fas-deficient T cells are not associated with the cell death defects in B6/*lpr* T cells came from experiments carried out in apoptosis-free conditions (Fig. 4, 5). In these circumstances, control T cells did not undergo cell death; the proliferative advantage of Fas-deficient T cells could therefore not be attributed to an increase in cell survival. Moreover, these results indicated that lack of Fas permits B6/*lpr* T cells to extend their proliferative response beyond the immediate reaction to TCR stimulation.

In another approach, we demonstrated that Fas/FasL interaction directly reduced proliferation of Fas-expressing, restimulated B6/*gld* T cells, independently of the Fas/FasL apoptosis-inducing effect (Fig. 6). The data showed that Fas/FasL interaction is directly responsible for reducing T cell proliferation, since FasL-deficient, Fas-expressing B6/*gld* T cells hyperproliferated after secondary challenge. These findings also suggest that the Fas pro-apoptotic and anti-proliferative functions are independent, and ultimately rely on Fas/FasL interaction and not the presence of Fas alone.

We ruled out the possibility that B6/*lpr* T cell hyperproliferation is due to differences in activation/memory status before second stimulation, or that culture conditions favor selection of Fas-deficient cell clones with initial high proliferative capacity (Fig. 7, 8). For that purpose, naïve (CD44low/CD62Lhigh) and memory (CD44high/CD62Llow) T cells were sorted and their proliferation and activation

was tested. Fas suppressed proliferation of apoptosis-resistant T cells after secondary stimulation, but did not affect primary T cell responses; in fact, it slightly enhanced stimulation of naïve T cells. The difference in the Fas effect on primary and secondary stimulation might be due to dissimilarities in the T cell response to activation in these two contexts. The Fas/FasL interaction after primary stimulation contributes to the proliferative response, offering necessary costimulation for naïve T cells (Alderson et al., 1993; Maksimow et al., 2006; Rethi et al., 2008). Our work shows that following secondary stimulation, Fas/FasL interaction provides negative regulation of the proliferative response.

We also observed that in both B6/*lpr* and B6 mice, CD44high/CD62Llow T cell proliferation was lower than that of CD44low/CD62Lhigh T cells. Other studies also reported that *in vivo*-generated memory T cells show characteristics of senescence, with diminished proliferative potential *in vitro* (Shimatani et al., 2009; Effros 2003; Di Mitri et al., 2011). Similarly, T cells from patients with chronic inflammation or long-standing infections respond poorly when stimulated *in vitro* with antigens or mitogens, although the mechanism underlying this hyporesponsivness is not known (Kiessling et al., 1996).

Overall, these data indicate that, in the absence of Fas, T cells show a hyperproliferation profile, and the proliferative advantage is not associated with cell death defects. In addition to its proapoptotic function, Fas therefore has another role in T cell homeostasis.

Fas regulation of proliferation is biologically significant

We generated B6/*lpr*-p21tg mice that overexpress p21 to test whether T cell hyperproliferation is responsible for lymphadenopathy development and lupus-like disease. p21 overexpression inhibited B6/*lpr* T cell hyperproliferation after secondary stimulation (Fig. 16), but had no effect on primary stimulation or on the B6/*lpr* apoptotic defect (Fig. 15). We therefore considered that B6/*lpr*-p21tg mice were an appropriate model to study the biological significance of Fas as a regulator of T cell proliferation. We found that p21 overexpression greatly reduced B6/*lpr* T cell hyperproliferation *in vivo* (Fig. 9) as well as lymphadenopathy (Fig. 11C), suggesting the biological relevance of the hyperproliferative effect of reactivated B6/*lpr* T cells. Our results also showed that p21 is a potent autoimmunity suppressor, as it can restrain autoimmunity and death in MRL/*lpr* mice, which develop an accelerated, severe form of lupus-like disease (Fig. 13, 14).

Overall our data indicate that the Fas proliferation-regulating effect has physiological importance, since p21 overexpression does not affect the B6/*lpr* apoptotic defect, but reduces lymphadenopathy by decreasing B6/*lpr* T cell hyperproliferation. This *in vivo* hyperproliferation suppression function

of Fas, together with its proapoptotic effect, is necessary for activated T cell homeostasis. Our findings suggest an alternative mechanism through which Fas deficiency leads to lymphadenopathy, and should be considered for understanding T cell expansion syndromes and diseases associated with Fas expression.

The role of Fas in the control of NF-κB and ERK1/2 activation

Fas deficiency leads to hyperproliferation of T cells after a second stimulation; in addition, lack of Fas results in hyperactivation of restimulated T cells, as determined by analysis of activation markers CD25 and CD69. To understand the molecular basis that underlies these intrinsically connected events, we analyzed two major signaling pathways involved in T cell proliferation after TCR-dependent activation, NF-κB and ERK1/2.

NF-κB has a fundamental role in regulating the immune response, as a key factor in transcription of the genes essential for cell proliferation and survival. We found that hyperproliferation of B6/*lpr* T cells 24 h after restimulation is associated with NF-κB hyperactivation (Fig. 18). The signaling pathway leading from TCR stimulation to NF-κB activation is not well defined, although the CBM complex, formed by CARMA-1, BCL-10 and MALT-1, is essential for NF κB nuclear translocation (Frischbutter et al., 2011; Egawa et al., 2003; Thome et al., 2003; Rueda et al., 2005; Sommer et al., 2005; Matsumoto et al., 2005). Following secondary stimulation of T cells, we detected enhanced expression of these proteins in B6/*lpr* T cells compared to controls (Fig. 23A, bottom). Caspase-8 is recruited to the CBM complex after TCR activation; in this molecular context, caspase-8 contributes to degradation of the NF-κB inhibitor IκBα and subsequent translocation of NF-κB dimers to the nucleus (Su et al., 2005; Bidere et al., 2006). NF-κB hyperactivation in B6/*lpr* T cells is caspase-8-dependent, since blockade of caspase-8 activity led to decreased activation of this transcription factor (Fig. 21C). Inhibition of NF-κB activation led to a reduction in the B6/*lpr* T cell proliferation, which confirms that hyperproliferation of these cells is, to a great extent, NF-κB activation-dependent. This was confirmed in the B6/*gld* model, in which inhibition of NF-κB activation by FasL treatment resulted in reduced B6/*gld* T cell proliferation after restimulation (Fig. 18C).

In addition to showing NF-κB hyperactivation in B6/*lpr* T cells, we demonstrated that Fas deficiency enhanced the ERK1/2 signaling pathway. Phospsho-rylation of ERK1/2 following secondary stimulation was earlier and stronger in B6/*lpr* T cells compared to controls, suggesting that ERK1/2 activation promotes proliferation after TCR restimulation (Fig. 24A). Our findings indicate that, after the second stimulation, Fas controls the T

cell proliferative response through negative regulation of the NF-κB and ERK1/2 signaling pathways.

Involvement of whole form caspase-8 in the B6/*lpr* T cell hyperproliferation

Caspase-8 is necessary not only for death receptor-mediated apoptosis, but is also essential for T cell activation (Chun et al., 2002; Salmena et al., 2003; Siegel 2006; Lamkanfi et al., 2007). Reports indicate the fundamental role of capase-8 in TCR-mediated NF-κB activation (Su et al., 2005; Dohrman et al., 2005; Biedre et al., 2006; Misra et al., 2007); in accordance with these observations, caspase-8 inhibition is associated with T cell proliferation defects and immune deficiency in humans and mice (Kennedy et al., 1999; Falk et al., 2004; Mistra et al., 2005). Both caspase-8-mediated NF-κB activation and apoptosis require enzymatic activity of caspase-8, although the molecular form of the protease that shows this activity is distinct for each of these processes. For the apoptotic cascade, caspase-8 autoprocessing is necessary, whereas the full form of active caspase mediates the NF-κB signaling pathway (Lamkanfi et al., 2007; Bidere et al., 2006). Some studies also indicate that the transition from the inactive to the active caspase-8 form does not involve Fas signaling (Misra et al., 2007; Dohrman et al., 2005a; Dohrman et al., 2005b).

Following primary TCR triggering, the entire caspase-8 molecule is essential for proliferative signaling, as this form promotes NF-κB activation (Scheme 4, Introduction; Bidere et al., 2006). Here we showed that active caspase-8 also has an important role in enhancing pro-proliferative signals after secondary stimulation. Using a biotin-VAD-fmk/streptavidin precipitation system, we detected active, unprocessed caspase-8 after secondary stimulation in both control and Fas-deficient T cells, although caspase-8 activation was much higher in B6/*lpr* T cells (Fig. 20A). Moreover, caspase-8 association with the CBM complex members was more efficient in Fas deficient T cells. Our experiments established that, after the second stimulation, full-length caspase-8 is more active in B6/*lpr* T cells than in controls, and that caspase-8 forms a part of a protein complex that tends to assemble lymphocyte proliferation-related signaling molecules more efficiently in B6/*lpr* than in B6 T cells. This complex is composed of the proteins known to participate in the signal transduction that leads to NF-κB activation, and includes CARMA1, BCL10, MALT1, FADD, Flip, and IKKα (Fig. 20A).

We confirmed that caspase-8 was indeed enzymatically active; a colorimetric assay showed an increase in caspase-8 enzymatic activity that coincided with hyperproliferation following the second stimulation in B6/*lpr* T cells (Fig. 20D).

Our results demonstrate that the caspase-8 activity needed for T cell proliferation is independent of Fas-mediated cell death signaling, since Fas expression is defective in B6/*lpr* T cells.

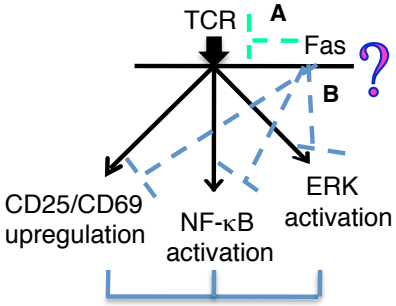
In accordance with these observations, we found that capase-8 activity is in fact involved in the B6/*lpr* T cell hyperproliferative phenotype, as inhibition of its enzymatic activity led to reduced NF-κB activation and decreased cell proliferation (Fig. 21). These findings implicate caspase-8 in Fas regulatory function of apoptosis-surviving T cell proliferation, due to its role in NF-κB activation.

Using confocal microscopy, we localized caspase-8 in B6/*lpr* T cells at the plasma membrane before and after secondary stimulation. In contrast, in B6 T cells caspase-8 staining was almost entirely cytoplasmic following restimulation (Fig. 19). This cytoplasmic fraction of caspase-8 is however not pro-apoptotic, since Lee et al. showed that zVAD blocs caspase-8 internalization and consistently inhibits apoptosis (Lee et al., 2006). This suggests that Fas/FasL interaction is required for internalization of the pro-proliferative portion of capsase-8. It is also tempting to speculate that Fas collaborates with caspase-8 in the control of T cell proliferation, possibly by mediating its translocation from the plasma membrane, where the protein complex required for TCR-mediated NF-κB activation, which includes CARMA-1, BCL10 and MALT-1, is located (Gaide et al., 2002; Dykstra et al., 2003).

TCR dependency of the B6/*lpr* hyperproliferative phenotype

We established that, after secondary stimulation, B6/*lpr* T cells are hyperactivated compared to controls. This phenotype is linked to enhanced activation of NF-κB and ERK1/2 and is caused by lack of negative control by the Fas/FasL interaction. Elevated proliferation of B6/*lpr* T cells is thus dependent on signaling pathways downstream of the TCR.

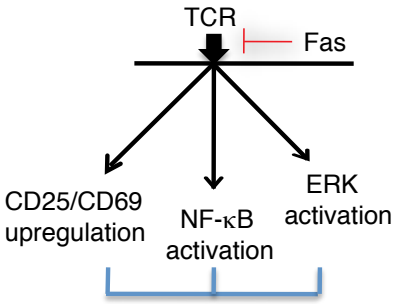
In Scheme 10, we illustrate our hypothesis wherein Fas acts in the control of proliferation after secondary stimulation by inhibiting TCR-dependent signaling pathways, or by interacting directly with the TCR complex (Scheme 10). In the latter case, the interaction would have an impact on all downstream signaling pathways that lead to T cell activation and proliferation. We found



Scheme 10. Scheme showing possible Fas effects on TCR-dependent signaling
A. Fas might interact directly with the TCR complex, thus affecting all TCR-triggered pathways, or (**B**) Fas might have a regulatory effect downstream of the TCR complex, affecting some or all of the pathways.

that hyperproliferation of B6/*lpr* T cells is a TCR-mediated phenomenon, since after the IL-2 expansion phase, only TCR-restimulated Fas-deficient T cells hyperproliferated compared to controls. Proliferation of B6/*lpr* T cells without TCR restimulation was not higher than that of control T cells (Fig. 24B). These data demonstrate that Fas interacts with proximal TCR signaling events to negatively regulate T cell proliferation. Indeed, Fas acts upstream of PKC and/or $Ca2^{+}$ signaling in the TCR proliferative pathway (Fig. 25A, B), and Fas deficiency influences early signaling steps in the proliferative pathway, including phosphorylation of Src, ZAP70, LAT and PKCα (Fig. 25C). Our results therefore indicate that, in the model we propose, Fas interacts directly with TCR complex and thus affects all downstream signaling pathways that lead to T cell activation and proliferation (Scheme 11).

Differences in the phosphorylation pattern of the proteins analyzed were apparent before secondary stimulation. These differences appear to have no functional significance, however, since there was no NF-κB activation, nor was proliferation of B6/*lpr* T cells higher than that of control T cells before restimulation or during the IL-2 expansion phase. This suggests that Fas is a regulator of the unengaged TCR complex and can therefore control initiation of TCR-mediated signaling directly. These findings concur with observations that showed pre-association of Lck and Fas in untreated cells and suggested that the unengaged TCR complex acts as a direct regulator of Fas signaling (Akimzhanov et al., 2010; Sharif-Askari et al., 2007). Our findings thus implicate Fas as a regulator of proximal TCR signaling events, and show that its deficiency leads to the pathological amplification of TCR-induced signaling.



Scheme 11. Fas affects the TCR complex
Fas interacts directly with the TCR complex and negatively regulates early signaling events, which influences all downstream pathways.

To maintain T cell homeostasis, a proliferative response should terminate once the antigen has been cleared. One mechanism that contributes to the signal arrest initiated by antigen binding is TCR downmodulation (Valitutti et al., 1996; Valitutti et al., 1997; Cai et al., 1997; Liu et al., 2000; Davanture et al., 2003). We found that TCR downregulation was defective in B6/*lpr* T cells (Fig. 26); persistent TCR expression in these cells could therefore lead to enhanced, prolonged signaling, which would result in the hyperproliferative phenotype. This could in turn be implicated in the loss of self-tolerance observed in B6/*lpr* mice. In contrast, TCR-induced signaling in control T cells is not maintained for such a

prolonged period, and thus the functional outcome is controlled proliferation and self tolerance. In a recent study, Katzman and colleagues indeed showed physiological relevance for the duration of TCR-induced signals; they reported that length of T cell-APC (antigen-presenting cell) interaction and concomitant TCR-mediated signaling are key determinants in lymphocyte tolerance vs. activation (Katzman et al., 2010). They suggested that loss of self tolerance is caused by prolonged T cell-APC interaction, which induces enhanced TCR signaling, whereas transient interactions and signaling lead to abortive activation that could account for the tolerant phenotype.

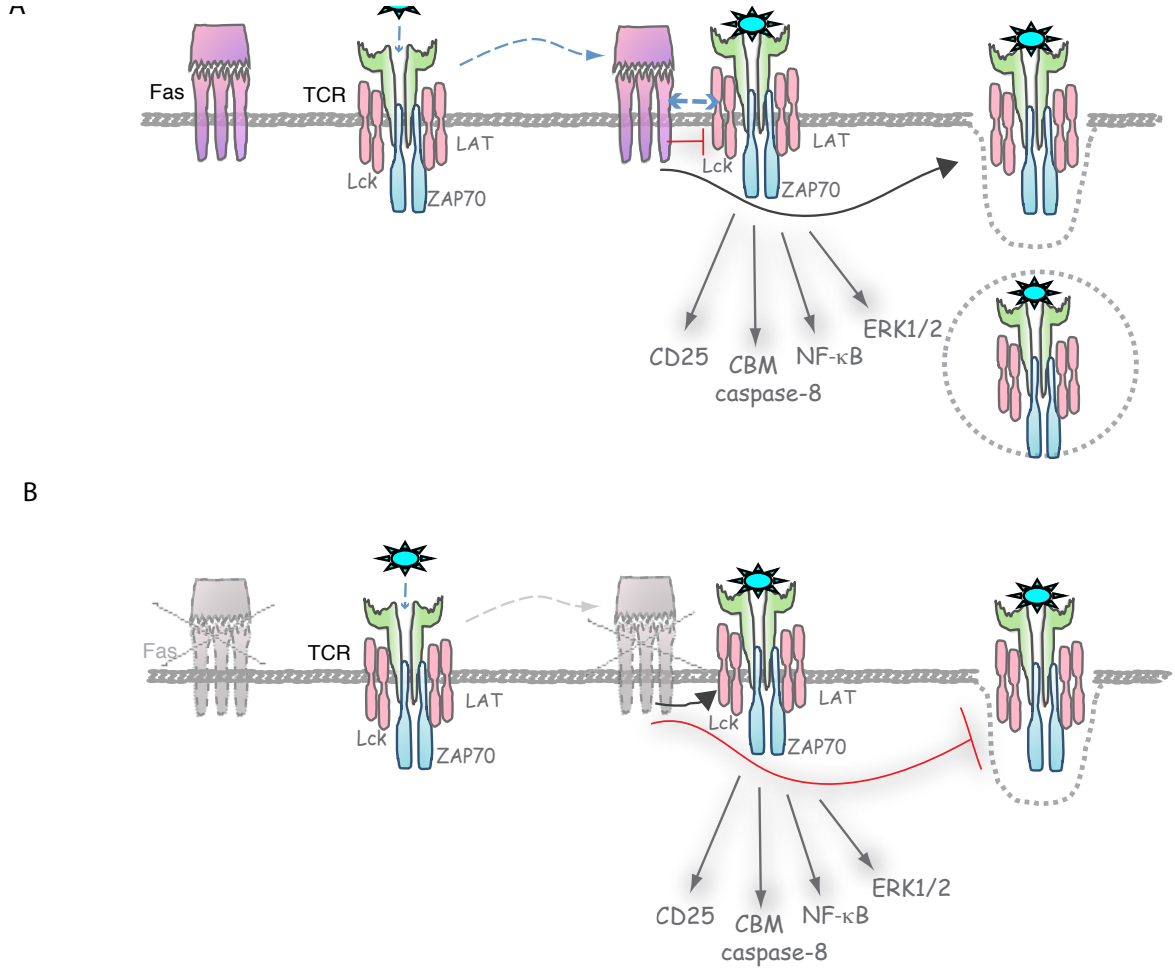
Overall, our data indicate that Fas is necessary for the regulation of early TCR signaling events, and that the greater proliferative capacity of B6/*lpr* T cells results from a direct effect of Fas deficiency on TCR expression. The absence of Fas impairs TCR downmodulation; this could result in persistent signaling that induces stable, prolonged expression of activation markers, and could in turn provoke a hyperproliferative phenotype and loss of self tolerance in B6/*lpr* T cells.

Fas is necessary for correct TCR internalization after secondary stimulation

Using a flow cytometry-based approach, we found that TCR downregulation was defective in Fas-deficient T cells after secondary stimulation (Fig. 26A, 27A). The TCR downmodulation process comprises receptor internalization, recycling, phosphorylation and degradation through the ubiquitin pathway (Alcover et al., 2000). We analyzed the TCR recycling ratio, and detected no difference between B6 control and B6/*lpr* T cells (Fig. 28D). In accordance with the similar proliferation rates for B6 and B6/*lpr* T cells, we detected no differences in the degree of receptor internalization after primary stimulation or during the IL-2 expansion phase (Fig. 27D, E). This observation supports the hypothesis that correct TCR internalization allows T cells to terminate signaling, and thus to end the proliferative response. Consistent with this hypothesis, we found that the TCR internalization process is impaired in B6/*lpr* T cells (Fig. 27C, 28C). Confocal microscopy analysis further supported the finding of defective surface TCR downregulation in B6/*lpr* T cells after the second stimulation (Fig. 27F). Our finding that B6 control and B6/*lpr* T cells have a similar TCR recycling ratio also indicates that defective internalization rather than receptor recycling ac-

counts for lack of TCR downmodulation in B6/*lpr* T cells. In addition, we demonstrated that impaired TCR internalization in Fas-deficient T cells is specific to the TCR and not to the general endocytosis pathway, since transferrin internalization was unaffected by the absence of Fas after restimulation (Fig. 28A, B).

In summary, our findings establish that Fas deficiency leads to inhibition of TCR internalization after secondary stimulation; this can result in B6/*lpr* T cell hyperactivation and hyperproliferation (Model 1).



Model 1. Fas is a negative regulator of early signaling events
A. Following secondary stimulation, Fas is recruited to the TCR complex, where it negatively regulates activation and further recruitment of signaling molecules involved in early signaling, such as Lck, ZAP70 and LAT. This allows controlled activation of downstream signaling pathways including ERK, CBM complex and NF-κB. In addition, Fas promotes internalization of the activated TCR complex, which results in signal termination and prevents hyperactivation of T cells. **B.** Absence of Fas during T cell restimulation leads, on one hand, to pathological amplification of TCR proximal signaling, resulting in hyperactivation of downstream pathways such as NF-κB and ERK; on the other hand, it inhibits TCR complex internalization, which leads to prolonged signaling and T cell hyperactivation.

Direct interaction of Fas with the TCR complex regulates T cell activation

Our results thus far indicate that Fas has an important regulatory role in T cell proliferation by interacting with signaling molecules involved in early events. We placed Fas action upstream of the PKC and/or $Ca2^{+}$ cascades in the TCR signaling pathway, and found that TCR stimulation triggered enhanced phosphorylation of upstream molecules in Fas-deficient T cells. Finally, we established that Fas is needed for correct TCR downregulation after secondary stimulation, and that lack of Fas leads to impaired internalization of the TCR.

In accordance with these data, confocal analyses showed that Fas co-localizes with the TCR in control T cells, before and after restimulation (Fig. 29). Such co-localization would allow a direct impact of Fas on TCR internalization and other TCR-triggered signaling events. Our data also showed that both Fas and TCR translocated from the plasma membrane to the cell interior after restimulation, which could reflect internalization triggered by antigen binding, as we did not observe similar translocation in B6/*lpr* T cells, which have defective TCR internalization (Fig. 29).

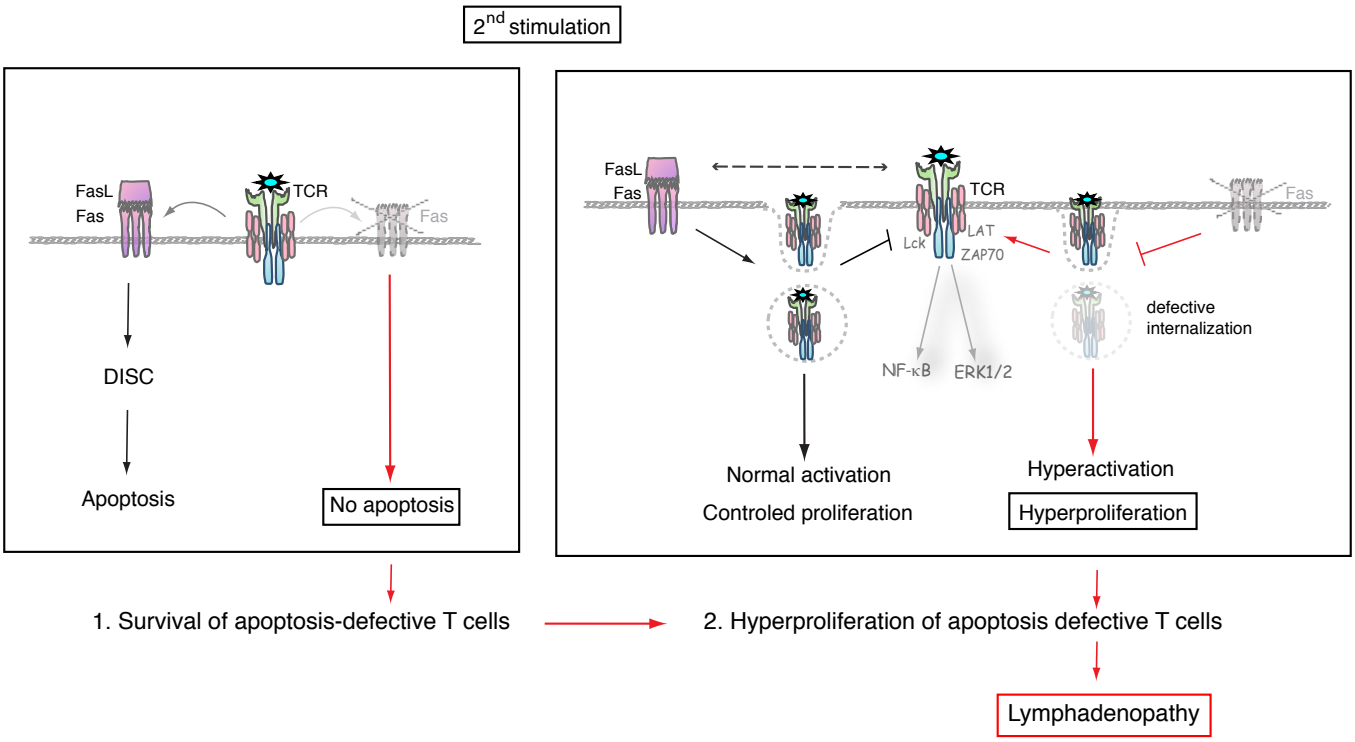
Consistent with Fas and TCR co-localization in B6 T cells, we detected direct interaction of Fas and TCR complex after secondary stimulation. Fas from B6 control T cells coimmunoprecipitated with TCR and with other proteins involved in early signaling; in B6/*lpr* T cells, however, Lck, ZAP70 and PKCθ recruitment to the TCR were considerably enhanced after restimulation in the absence of Fas (Fig. 30). These results are in line with the recent report of Akimzhanov et al. showing Fas and TCR interaction for the initiation of Fas-mediated apoptosis (Akimzhanov et al. 2010). In their experiments, Fas bound directly to and activated TCR components Lck and PLC-γ1, suggesting that TCR is a potent regulator of Fas signaling. Our study indicates that this interaction is bidirectional, since Fas is a key regulator of secondary T cell activation and proliferation.

These data suggest a model wherein Fas recruitment to the TCR following restimulation leads to impairment in the assembly of proteins important in TCR-activated signaling pathways. Lack of this blockade could result in hyperactivated proliferative signaling, as is the case in Fas-deficient B6/*lpr* T cells. Fas association with the TCR thus appears to be critical in the control of proximal TCR signaling events. It would be of interest to define the signaling event(s) that triggers Fas/TCR association. Better understanding of the precise molecular pathway activated by TCR in Fas-deficient mice promises to shed light on various unexplained aspects of autoimmunity, as well as on normal immune function.

Final conclusion and Model

In conclusion, we show that Fas attenuates proliferation of apoptosis-surviving B6 T cells. Our data establish an essential Fas function in the control of T cell proliferation, independent of the apoptosis-inducing potential of Fas. We provide evidence that Fas has an important role in the regulation of proximal TCR signaling events, including phosphorylation of the upstream molecules Lck, ZAP70, LAT and PKC, and internalization of the TCR. As a consequence, Fas controls downstream signaling pathways that mediate T cell proliferation and activation, such ERK1/2 and NF-κB.

Based on these observations, we suggest that Fas/FasL signaling controls T cell homeostasis and lymphadenopathy development through two distinct functions; first, by promoting apoptosis, and second, by regulating proliferation of apoptosis-surviving T cells (Model 2). This dual function underlies the potent homeostatic role of Fas in the immune system.



Model 2. Dual role of Fas in the homeostasis of activated T cells
Fas induces apoptosis of reactivated T cells, and its absence leads to accumulation of apoptosis-defective T cells. Moreover, Fas controls proliferation of apoptosis-surviving T cells; it promotes activated TCR complex internalization and thus inhibits early signaling, which allows controlled T cell activation and proliferation. Fas deficiency leads to inhibition of Fas internalization and to amplification of early signaling and downstream pathways, resulting in T cell hyperactivation and hyperproliferation. Defective apoptosis, together with hyperprolferation of apoptosis-surviving T cells, leads to development of lymphadenopathy.

1. In addition to its pro-apoptotic role, Fas has an alternative function in T cell homeostasis by controlling T cell activation and proliferation. The Fas proliferation-regulating effect is physiologically relevant, since p21 overexpression does not affect the B6/*lpr* apoptotic defect, but reduces lymphadenopathy by decreasing B6/*lpr* T cell hyperproliferation.

2. Defective Fas signaling results in hyperproliferation of TCR-rechallenged B6/*lpr* T cells *in vitro*, suggesting an important anti-proliferative function for Fas after secondary stimulation, independent of its proapoptotic role.

3. Fas interaction with its ligand is essential for the inhibition of hyperproliferation after restimulation, but this interaction is independent of apoptosis induction.

4. Hyperproliferation of B6/*lpr* T cells results from their hyperactivation. Fas acts as a negative regulator of T cell activation and proliferation by negatively controlling proximal TCR signaling events as well as activation of the CBM complex, NF-κB and ERK1/2 pathways.

5. Correct TCR internalization is essential for signaling termination and proliferation control. TCR downregulation in B6/*lpr* T cells is defective due to impaired TCR internalization.

6. Fas interacts directly with the TCR complex in the negative control of T cell activation and proliferation.

7. The anti-proliferative function of Fas, together with apoptosis induction, are fundamental in the control of immunological homeostasis and peripheral tolerance.

Introducción

En el proceso de una respuesta inmune *in vivo*, el encuentro de las células T con un antígeno se ve acompañado de una rápida proliferación clonal, seguida de una reducción en el número de células, alcanzándose finalmente un equilibrio. Se pensaba que la apoptosis mediada por Fas, un miembro de la familia de receptores del factor de necrosis tumoral (TNF del inglés Tumor Necrosis Factor), es el principal mecanismo de esta retracción del sistema inmune, llamada fase de contracción. Sin embargo, la regulación *in vivo* de este proceso no esta definida en detalle. Las células que sobreviven constituyen la población de células T de memoria. La des-regulación de la respuesta proliferativa de las células T o de la apoptosis, puede tener severas consecuencias en la homeostasis del sistema inmune.

La tolerancia es un elemento crítico del sistema inmune ya que asegura su capacidad de reconocer y responder sólo a antígenos exógenos y no a los propios del organismo. La pérdida de la capacidad de distinguir entre lo propio y lo no propio conduce a respuestas inmunes contra autoantígenos y al desarrollo de enfermedades autoinmunes. La tolerancia contra antígenos propios, se mantiene a través del proceso de tolerancia central que ocurre durante el proceso de maduración en el timo y procesos periféricos como la "ignorancia" inmunológica, delección, inhibición o supresión de clones autoreactivos.

Se sabe que la eliminación de las células T activadas es el principal mecanismo para asegurar la tolerancia en periferia. La interacción de Fas con Fas ligando (FasL), es fundamental para la homeostasis de células T y la inducción de tolerancia.

La muerte celular inducida por activación (AICD del inglés Activation-Induced Cell Death) es el proceso *in vitro* de inducción de muerte celular en células T activadas por una re-estimulación del TCR. En este modelo experimental, se estimula inicialmente las células T nativas con antígenos o mitógenos; seguidamente estas células son expandidas en un medio de cultivo con IL-2 y re-estimuladas vía TCR. Tras la re-estimulación, se da una inducción del FasL y su unión a Fas, induce cambios conformacionales en el receptor que permiten el reclutamiento de la molécula adaptadora FADD (del inglés Fas-Associated Death Domain protein). Esto conduce al reclutamiento del iniciador caspasa-8 y la formación del complejo de señalización inductor de muerte (DISC del inglés Death Inducing Signaling Complex). La internalización del receptor asociado a DISC, permite

el auto-procesado de la caspasa-8 y su activación, disparando la apoptosis.

La caspasa-8 en su forma completa (sin procesar) está asociada con la proliferación de los linfocitos tras estimulación del TCR, y su actividad es necesaria para la activación de NF-κB. Se ha descrito que la caspasa-8 se recluta al complejo CBM (CARMA1, BCL10, MALT1) y en este contexto molecular, contribuye a la activación del complejo IKK. La inhibición *in vivo* e *in vitro* de la actividad de caspasa-8 en las células T CD4+ tanto humanas como de ratón, conduce a una menor proliferación y producción de IL-2 después de una estimulación por TCR, constituyendo así la caspasa-8 un elemento indispensable para la proliferación normal de los linfocitos.

La activación de células T, su proliferación y sus funciones inmunológicas son el resultado de eventos de señalización que se originan a partir de la estimulación del receptor de células T (TCR del inglés T Cell Receptor). Cuando el TCR reconoce su ligando, se activa la fosforilación de residuos de tirosina en los motivos ITAMs (del inglés Immunoreceptor Tyrosine-based Activation Motifs) de las cadenas TCR-ζ y CD3-γ, -δ y –ε, por las kinasas de la familia src, Lck y en menor grado Fyn. Esto conduce al reclutamiento y fosforilación de ZAP70, que a su vez, recluta y fosforila a moléculas adaptadoras como LAT, SLAP, SLP-76, PLC-γ1 y Cbl. Seguidamente, se activan múltiples cascadas de señalización incluyendo ERK, JNK, NF-κB y NFAT, que en último término inducen las funciones efectoras.

Uno de los procesos cruciales en el apagado de la señalización por TCR es su propia internalización y subsecuente degradación. El complejo TCR se internaliza a través de vesículas cubiertas por clatrina y existen al menos dos rutas distintas de endocitosis del TCR. Una es dependiente de la actividad tirosina kinasa, que conduce a la ubiquitinación del TCR; la otra, es dependiente de la activación mediada por la proteína kinasa C (PKC) del dominio di-leucina de la subunidad CD3-γ, que origina el reciclado del receptor. Se ha sugerido que la función más probable de la reducción del TCR en la superficie celular, es proteger a las células de una sobre-estimulación y que este proceso de modulación es una eficiente manera de inducir tolerancia.

Una deficiente señalización mediada por Fas/FasL en ratones B6/*lpr* y en pacientes con síndrome linfoproliferativo autoinmune (ALPS del inglés Autoimmune LymphoProliferative Syndrome) origina el desarrollo de linfoadenopatías y autoinmunidad caracterizadas por la acumulación de células T Dobles Negativas (DN), células T de memoria, niveles elevados en suero de IgGs y auto-anticuerpos, formación de complejos inmunes en tejidos y glomerulonefritis leve.

La pérdida de apoptosis inducida por Fas en las células T activadas, se ha sugerido como causa directa de los fenotipos mostrados por ratones B6/*lpr* y en los pacientes del ALPS. Defectos en apoptosis sin embargo, son insuficientes para justificar la linfoadenopatía y la autoinmunidad observada en ratones B6/

lpr, ya que la ausencia de otras moléculas pro-apoptóticas como FADD y caspasa-8 originan un defecto en apoptosis pero no manifestaciones patológicas como las descritas anteriormente. Además, a pesar del bien conocido papel en la muerte celular de Fas *in vitro*, la relevancia *in vivo* de la apoptosis inducida por Fas es materia de debate.

Además del desarrollo de la enfermedad autoinmune en pacientes con ALPS y ratones B6/*lpr*, las células T de sus ganglios linfáticos hiper-proliferan *in vivo*, lo cual está aún por explicar. Tal y como se mostrará en esta tesis, esta hiper-proliferación es relevante fisiológicamente, ya que su inhibición origina una reducción de la linfoadenopatía; como en el caso de los ratones B6/*lpr* que sobreexpresan el inhibidor de ciclo celular p21en sus células T.

Objetivos

La enfermedad autoinmune en los ratones B6/*lpr* es el resultado de una alteración severa de la homeostasis inmune. Ya que defectos en apoptosis debidos a la deficiencia de Fas no pueden explicar el desarrollo de la linfoadenopatía, la hiper-activación o la hiper-proliferación de las células T de estos animales, el objetivo de esta tesis es mostrar que, además de la inducción de apoptosis, Fas tiene otras funciones en la homeostasis inmune, que están asociadas con el control de la activación de células T y su proliferación. Además, analizamos el efecto de la sobre-expresión del inhibidor de ciclo celular p21 en la hiper-proliferación observada en los ratones B6/*lpr*.

Establecimos los siguientes objetivos:

1. Analizar la capacidad proliferativa de las células T deficientes en Fas y FasL y su estatus de activación de NF-κB

2. Estudiar la importancia de la interacción Fas-FasL y el mecanismo molecular que subyace en el efecto inhibitorio de Fas en la proliferación secundaria de las células T

3. Determinar en qué punto de la señalización implicada en la activación / proliferación, ejerce Fas su papel inhibidor

4. Identificar la relación de Fas con el TCR en la activación y proliferación de las células T

5. Intentar inhibir la hiper-proliferación mostrada en ratones B6/*lpr*

6.Analizar el efecto *in vivo* de la sobre-expresión de p21 en las células T de ratones B6/*l*

Resultados

En esta tesis mostramos que además de su efecto pro-apoptótico, Fas es un regulador crítico de la activación y proliferación de las células T que sobreviven a la apoptosis. Tras re-estimulación, las células T B6/*lpr* alcanzan un mayor estado de activación e hiper-proliferan comparadas con las células control. Esta hiper-proliferación es biológicamente relevante, ya que su inhibición por la sobre-expresión de p21 reduce la linfoadenopatía y las manifestaciones autoinmunes de ratones deficientes en Fas.

El control por Fas de la proliferación es independiente de su potencial inductor de AICD, ya que el fenotipo hiper-proliferativo se observa también en condiciones sin apoptosis. Además, la interacción de Fas con FasL recombinante atenúa directamente la hiper-proliferación de células T B6/*gld* deficientes en FasL, re-estimuladas en presencia del inhibidor de muerte celular.

Nuestro análisis de la respuesta proliferativa tanto de células T nativas como de memoria, excluyó la posibilidad de que la hiper-proliferación de células T B6/*lpr* fuera debida a diferencias en el estatus activación / memoria antes de la segunda estimulación, o de condiciones artefactuales de cultivo que favorezcan la selección de clones deficientes en Fas con mayor capacidad proliferativa inicial.

Los resultados también implican que la ausencia de Fas en las células

T re-estimuladas conduce a una mayor activación de las cascadas de señalización NF-κB y ERK 1/2 y que Fas controla la activación y proliferación de células T mediante regulación negativa de la actividad de caspasa-8 en su forma completa. Caspasa-8 sin procesar controla la activación del complejo CBM, y la proliferación de las células T re-estimuladas, tanto en B6 como en B6/*lpr*, independiente de su función pro-apoptótica. Además, encontramos que tras re-estimulación del TCR en condiciones libres de apoptosis, caspasa-8 se localiza en diferentes regiones celulares en las células T deficientes en Fas que en controles. Esto sugiere que Fas es necesario para la internalización de la porción pro-proliferativa de caspasa-8.

Nuestros datos también indican que Fas es un regulador de los eventos proximales de la señalización del TCR, incluyendo la fosforilación de Lck, ZAP70 y PKC; la deficiencia de Fas conduce a una amplificación de estas señales. Además, la ausencia de Fas durante la re-estimulación de células T, resulta en la inhibición de la internalización del TCR. Una internalización deficiente del TCR en células T B6/*lpr* podría resultar en una activación prolongada de las cascadas de señalización, originando una hiper-activación de NF-κB y ERK 1/2, y finalmente, la hiper-activación e hiper-proliferación de las células T.

Vimos que en las células T control, Fas co-localiza con el TCR antes y después de una segunda estimulación, lo cual podría permitir una influencia directa de Fas en la internalización del TCR y otras señales dependientes del mismo. Además, Fas es reclutado al complejo TCR después de una re-estimulación, posiblemente impidiendo la agregación de proteínas importantes en las cascadas de señalización iniciadas por el TCR; la pérdida de este impedimento podría por otra parte, resultar en señales proliferativas hiper-activadas, como es el caso en células T B6/*lpr* deficientes en Fas.

Conclusiones

1. Fas, además de un papel pro-apoptótico, tiene una función alternativa en la homeostasis de las células T, controlando su activación y proliferación. El efecto regulador de proliferación de Fas es fisiológicamente relevante, ya que la sobre-expresión de p21 no afecta al defecto apoptótico de las células B6/*lpr*, pero reduce la linfoadenopatía que muestran los ratones B6/*lpr*, mediante la reducción de la hiper-proliferación de sus células T.

2. La señalización deficiente por Fas, resulta en hiper-proliferación de células T B6/*lpr* *in vitro* re-estimuladas vía TCR, sugiriendo una importante función anti-proliferativa de Fas tras una estimulación secundaria, e independiente de su rol pro-apoptótico.

3. La interacción de Fas con su ligando es esencial para la inhibición de la hiper-proliferación tras re-estimulación, pero este fenómeno es independiente de la inducción de apoptosis.

4. La hiper-proliferación de las células T B6/*lpr* resulta de su hiper-activación. Fas actúa como un regulador negativo de la activación y proliferación de células T mediante el control negativo de eventos proximales en la señalización del TCR, así como de la activación del complejo CBM, NF-κB y ERK 1/2.

5. Una correcta internalización del TCR es esencial para la terminación de la señalización y el control de la proliferación. La reducción del TCR en la superficie de las células T B6/*lpr* es defectuosa debido a una internalización impedida.

6.Fas interacciona directamente con el complejo TCR en el control negativo de la activación y proliferación de células T.

7. La función anti-proliferativa de Fas, junto con la inducción de apoptosis, son fundamentales en el control de la homeostasis inmunológica y tolerancia periférica.

En esta tesis, examinamos la naturaleza de las células T deficientes en Fas hiper-proliferativas y estudiamos su implicación en una enfermedad tipo lupus en ratones B6/*lpr*. Hemos visto que la reducción de la hiper-proliferación de células T, de hecho, atenúa las manifestaciones de la enfermedad en estos animales, e identifica a Fas como un potente regulador negativo de la proliferación de las células T pre-activadas. Nuestros datos indican que Fas se asocia con el complejo TCR y afecta a su inter-nalización así como a otros eventos de señalización tempranos. Basados en estos descubrimientos, proponemos un mecanismo de homeostasis de las células T que implica esta alternativa a la función pro-apoptótica de Fas.

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